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Anaerobic killing of mucoid *Pseudomonas aeruginosa* by acidified nitrite derivatives under cystic fibrosis airway conditions

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Mucoid, *muca* mutant *Pseudomonas aeruginosa* cause chronic lung infections in cystic fibrosis (CF) patients and are refractory to phagocytosis and antibiotics. Here we show that mucoid bacteria perish during anaerobic exposure to 15 mM nitrite (NO₂⁻) at pH 6.5, which mimics CF airway mucus. Killing required a pH lower than 7, implicating formation of nitrous acid (HNO₂) and NO, that adds NO equivalents to cellular molecules. Eighty-seven percent of CF isolates possessed *muca* mutations and were killed by HNO₂ (3-log reduction in 4 days). Furthermore, antibiotic-resistant strains determined were also equally sensitive to HNO₂. More importantly, HNO₂ killed mucoid bacteria (a) in anaerobic biofilms; (b) in vitro in ultrasupernatants of airway secretions derived from explanted CF patient lungs; and (c) in mouse lungs in vivo in a pH-dependent fashion, with no organisms remaining after daily exposure to HNO₂ for 16 days. HNO₂ at these levels of acidity and NO₂⁻ also had no adverse effects on cultured human airway epithelia in vitro. In summary, selective killing by HNO₂ may provide novel insights into the important clinical goal of eradicating mucoid *P. aeruginosa* from the CF airways.

Introduction

Pseudomonas aeruginosa is an important pathogen that is most refractory to therapy when it forms biofilms in the airways of cystic fibrosis (CF) patients. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Although airway CFTR functions predominantly as an epithelial Cl⁻ channel, it serves to coordinate Na⁺ absorption and Cl⁻ secretion to produce sufficient airway surface liquid (ASL) for normal mucus clearance (1). Without functional CFTR, isotonic hyperabsorption of ASL, driven by enhanced absorption of Na⁺ via the epithelial sodium channel, results in ASL depletion, mucus concentration, and the formation of stagnant mucus plaques (2). Another feature of CF ion transport dysfunction is that the ASL may be acidified (pH <6.5) due to defective bicarbonate (HCO₃⁻) ion transport (3).

Two simultaneous seminal studies have recently indicated that the mucus layer lining the CF airway lumen is anaerobic

and that robust biofilm formation by *P. aeruginosa* occurs under such conditions (4, 5). The anaerobic nature of the CF airway mucus reflects the oxygen-consuming activities of airway epithelia, *P. aeruginosa*, and other opportunistic pathogens as well as neutrophils that combat infection. As chronic CF lung disease progresses, mucoid, alginate-overproducing strains emerge and become the predominant form (6). Mucoid *P. aeruginosa* biofilms are inherently resistant to antibiotics (7) and phagocytic neutrophils (8). Although several gene products have been reported to stimulate either a genotypic or phenotypic switch to the mucoid form, the best-characterized mechanism of mucoid conversion in CF isolates is via mutations in *muca*, encoding an anti-σ factor (9). Without Muca, the extracytoplasmic σ factor AlgT(U) transcribes genes involved in alginate biosynthesis. Mutations in *muca* and mucoid conversion can be triggered in vitro when biofilms are treated with H₂O₂ at levels similar to those generated by human neutrophils (10), professional phagocytes that are abundant in the CF airways. Approximately 84% of mucoid CF isolates (*n* = 53) in the US have been shown to possess *muca* mutations (11). In contrast, mucoid *muca* mutant bacteria are found in approximately 44% of the CF isolates from Australia, although the number of patients studied was substantially less than in the US cohort (12).

An important link between mucoidy and anaerobic metabolism by *P. aeruginosa* was identified in a study demonstrating that mucoid organisms were incapable of reversion to their nonmucoid,

Nonstandard abbreviations used: ASL, airway surface liquid; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide; CF, cystic fibrosis; CI, competitive index; LB, Luria-Bertani broth; NAR, nitrate reductase; NIR, nitrite reductase; NOR, NO reductase.

Conflict of interest: A patent has been filed on this technology by the University of Cincinnati naming D.J. Hassett as the sole inventor. He would share in royalties, if any, according to the university's patent policy set forth in university rule no. 3361:10-19-01.

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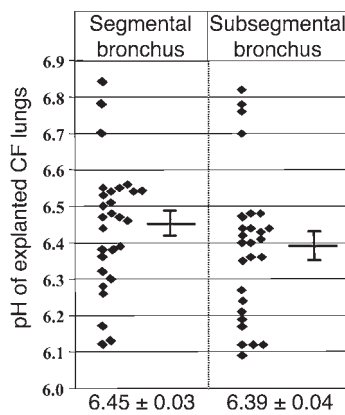


Figure 1

The pH of CF airway mucus is slightly acidic. In situ pH measurements of mucopurulent airway secretions from CF airways were made as described in Methods. Each measurement is presented as an individual data point in the scatter plots. The mean \pm SEM is shown below each panel.

antibiotic- and phagocyte-susceptible counterparts during anaerobic growth (13), results that were confirmed in 2002 by Wyckoff et al. (14). Recently, Worlitzsch and colleagues reported that anaerobic ASL favored production of alginate by *P. aeruginosa* (4). *P. aeruginosa* is capable of robust anaerobic growth by respiration using nitrate (NO_3^-) or nitrite (NO_2^-) as terminal electron acceptors (5). NO_3^- and NO_2^- are present in CF ASL (15–18) and sputum (19), and NO_3^- levels have been estimated as high as 600 μM (19), concentrations permissive for anaerobic *P. aeruginosa* growth in vitro and in vivo (20). Still, during anaerobic growth, *P. aeruginosa* must control the levels of a toxic intermediate of NO_2^- reduction, NO, by synthesis of protective NO reductase (NOR) (5). This requirement was demonstrated by the observation that overproduction of NO by anaerobic *P. aeruginosa* biofilms lacking the *rhl* quorum sensing circuit caused a metabolic suicide of these bacteria, an event that was prevented by a NO scavenger (21).

NO is also produced in normal airway epithelia by 3 different NO synthases (NOSs). Neuronal NOS and eNOS isoforms are constitutively expressed. The third isoform (iNOS) is also constitutively expressed in the airways but is inducible and upregulated in response to proinflammatory cytokines and bacterial LPS (22). Specifically, the iNOS2 class of enzymes contributes most effectively to the antimicrobial armament of the airway. However, in chronic CF, iNOS2 activity is significantly reduced (23), and this defect is thought to contribute to the persistence of airway *P. aeruginosa* populations.

Herein we describe a novel approach to killing mucoid *P. aeruginosa*. This is attributable in part to the organism's extremely low nitrite reductase (NIR) and NOR activity, a defect dependent upon *mutA* mutations. We investigated the mechanism of this effect in in vitro human studies and explored the therapeutic potential of NO_2^- in both human and mouse safety and mouse in vivo efficacy studies. Treatment of mucoid, *mutA* mutant bacteria with NO_2^- at pH 6.5 under anaerobic conditions, similar to conditions within mucopurulent secretions in the airways of CF patients, led to the death of these organisms. Ultimately, this observation may lead to the development of novel therapies for CF patients colonized with mucoid *P. aeruginosa*.

Results

CF airway luminal secretion pH is slightly acidic. The goal of this study was to test the hypothesis that mucoid *P. aeruginosa* are far more adept at growing during anaerobic respiration than nonmucoid bacteria, which is based on the fact that mucoid bacteria emerge and predominate during the chronic stages of CF airway disease. However, to accurately test this hypothesis, we wanted to assure that our medium pH was identical to that of the CF airway mucus. Previous in vitro studies suggest that the pH of the CF ASL is less than 6.5 (3). However, it is conceivable that the pH of mucopurulent secretions within CF airways might differ in vivo. Hence, we performed in situ pH measurements of luminal secretions from freshly explanted lungs removed from 9 different CF patients at the time of transplantation. The pH of the secretions was slightly lower than what was observed in vitro: 6.45 ± 0.03 in segmental airways and somewhat lower in more distal subsegmental bronchi (6.39 ± 0.04 ; Figure 1). These results guided our selection of pH 6.5 for subsequent experiments.

Mucoid, mutA mutant bacteria are sensitive to acidified NO_2^- . Based upon the slightly acidic pH measurements of segmental and subsegmental bronchi from CF transplant patients discussed above, we elected to grow various well-characterized *P. aeruginosa* strains at pH 6.5 under strict anaerobic conditions. Thus, upon anaerobic culture of *P. aeruginosa* at pH 6.5 with 15 mM NO_3^- (electron acceptor), mucoid *P. aeruginosa* strain FRD1 grew more slowly than nonmucoid strains PAO1 and FRD1/*pmucA*. Strain FRD1 is the best characterized mucoid, *mutA* mutant derived from a CF patient (24). However, no difference in viability patterns was observed (Figure 2A). Using 15 mM NO_2^- , however, mucoid FRD1 was killed at a rate of approximately 90% per day, while nonmucoid strains PAO1 and FRD1/*pmucA* remained viable over the 4-day incubation (Figure 2B).

After discovering this unique NO_2^- sensitivity of *mutA* mutant strain FRD1, we also found that NO_2^- killed these bacteria more effectively at a lower pH (Figure 2C), while little or no killing was observed in strain FRD1/*pmucA* at pH 6.0–7.5 (Figure 2C).

To test whether NO_2^- killing of mucoid bacteria occurs in the presence of nonmucoid bacteria, mucoid and nonmucoid *P. aeruginosa* were mixed and treated with 15 mM NO_2^- anaerobically. Strain FRD1 consistently lost viability at 3 different bacterial test ratios, while nonmucoid FRD1/*pmucA* maintained viability (Figure 2D).

We next characterized the dose-effect relationship between NO_2^- concentration and killing of mucoid bacteria. Figure 2E shows that 90–95% of the bacteria were killed by 15 mM NO_2^- and the LD_{50} was approximately 3 mM NO_2^- after 24 hours. The remaining organisms did not develop resistance to NO_2^- when we extended our study to 12 days (Figure 2F), demonstrating that all organisms were killed during this time period.

Other mucoid, mutA mutant CF isolates are also sensitive to NO_2^- . To test whether NO_2^- sensitivity is a trait of all *mutA* mutant mucoid CF isolates, the *mutA* genes of 94 mucoid clinical isolates recovered from a variety of CF clinics in the US and Canada were sequenced. Of 94 strains, 82 harbored *mutA* mutations, leading to either premature termination of translation (88%) or a loss of the stop codon (12%), thereby confirming previous findings (13) that *mutA* mutations are the major reason for mucoid conversion in CF isolates (Figure 3 and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI24684DS1). The most abundant mutations were

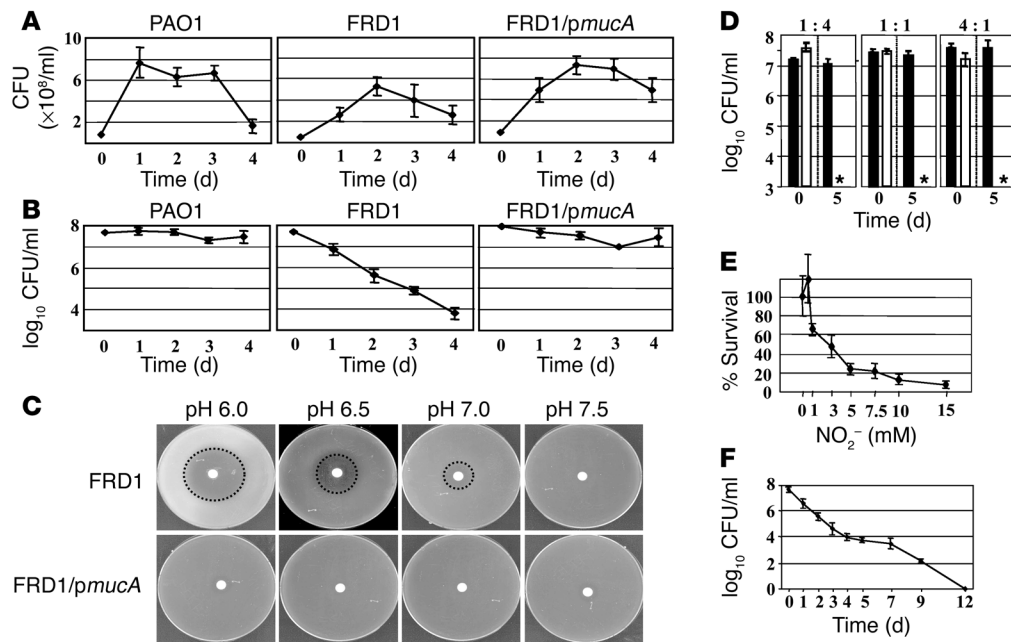


Figure 2 Mucooid *P. aeruginosa* FRD1 is selectively killed by NO₂⁻ in a pH-dependent manner. Anaerobic growth of *P. aeruginosa* strains at pH 6.5 using 15 mM NO₃⁻ or NO₂⁻ as a terminal electron acceptor. Aerobic overnight suspensions of PAO1, FRD1, and FRD1/*pmucA* were diluted 100-fold for the main anaerobic culture with NO₃⁻ (A) or NO₂⁻ (B). CFUs were enumerated each day and plotted as linear (A) and logarithmic (B) graphs. (C) NO₂⁻ sensitivity versus pH. FRD1 or FRD1/*pmucA* were seeded onto LB agar buffered at the indicated pH value. After placing a filter disk containing 10 μl of 1 M NO₂⁻, the plates were incubated anaerobically for 48 hours and scanned for viewing the zone of killing. To support anaerobic growth, 15 mM NO₃⁻ was included in the media. (D) Mucooid strain FRD1 (gray bars) and nonmucooid FRD1/*pmucA* (black bars) were incubated together at the indicated ratios (FRD1/*pmucA*:FRD1) for 5 days in the presence of 15 mM NO₂⁻, pH 6.5, after which CFUs were determined. **P* < 0.001 versus CFU in the initial inoculum. (E) Dose-response killing of mucooid strain FRD1 by NO₂⁻. Bacteria were suspended in LB (pH 6.5) with various amounts of NO₂⁻ for 24 hours under anaerobic conditions. Survival against NO₂⁻ is presented as the percentage of CFU relative to that in initial inoculum. (F) Long-term anaerobic exposure of mucooid strain FRD1 to 15 mM NO₂⁻ at pH 6.5. All experiments were performed in triplicate and presented as mean ± SEM.

single-bp deletions that resulted in a frame shift leading to premature termination of translation at bp 441 (the wild-type *mucA* gene is 585 bp; see Supplemental Table 1 for a detailed breakdown of *mucA* mutations in each strain). Consistent with the results of Martin et al. (9), approximately 13% of mucooid isolates had a wild-type *mucA* allele, indicating the presence of other mechanisms or mutations allowing for mucooid conversion. Upon anaerobic treatment with 15 mM NO₂⁻ at pH 6.5, almost all of the *mucA* mutant mucooid isolates (78 of 82 strains) showed increased susceptibility to NO₂⁻, with 74% killed by more than 2 logs relative to the mean of the nonmucooid *MucA*-proficient organisms (Figure 3). Importantly, 4 strains that were deemed antibiotic resistant were still sensitive to anaerobic treatment of acidified NO₂⁻ (Figure 3, arrows). Out of 12, however, 8 mucooid isolates with a wild-type *mucA* allele were resistant to acidified NO₂⁻. These results suggest that NO₂⁻ sensitivity is likely caused by *mucA* mutations and not by cellular processes associated with alginate overproduction.

In a separate longitudinal study using *P. aeruginosa* strains isolated from 5 different young CF patients, we consistently detected mucooid *P. aeruginosa* as the patient aged, a hallmark of chronic infection (Table 1). As predicted, initial airway colonization of each

patient was by nonmucooid *P. aeruginosa*. Most importantly, however, mucooid variants that were detected in patients A, B, C, and D possessed mutated *mucA* genes and were all killed by 15 mM NO₂⁻ (1.9–3.1 logs). These results indicate that the genotypic and phenotypic switch to the mucooid form that is sensitive to NO₂⁻ treatment can occur in patients less than 3 years of age (see patient C).

mucA mutations are responsible for NO₂⁻ sensitivity. The above results suggest that *mucA* mutations could be responsible for the enhanced sensitivity to NO₂⁻. Since *mucA* mutant bacteria overproduce alginate, we initiated experiments to test whether NO₂⁻ sensitivity is caused by *mucA* mutations and not by alginate production. An isogenic PAO1 *mucA* mutant, PDO300, whose intact *mucA* allele was replaced with that of strain FRD1 (*mucA22*) was also sensitive to NO₂⁻ (Table 2). Two FRD1 derived nonmucooid mutants [Δ *algD*, lacking GDP-mannose dehydrogenase, and Δ *algT(U)*, lacking AlgT(U)] were equally sensitive to killing by NO₂⁻ (Table 2). We next tested whether NO₂⁻ also killed *mucB*, *mucD*, and *algW* mutants of strain PAO1. Other than *mucA*, the aforementioned genes are the only reported loci that, when inactivated, allow for mucooid conversion in *P. aeruginosa* (25–27). In contrast to *mucA* mutant bacteria, these mutants were not sensitive to NO₂⁻ (Table 2). Most critically, 5 nonmucooid revertants of mucooid strains were still sensitive to HNO₂ (see Table 2). Therefore, our results suggest that NO₂⁻ sensitivity is *MucA*- and not alginate dependent. Finally, the LD₅₀ of NO₂⁻ for sensitive strains was almost identical to that for FRD1 (Figure 2E), suggesting that the rate at which these organisms were killed by NO₂⁻ was similar to that of strain FRD1.

HNO₂ is required for killing of *mucA* mutant bacteria; NO and other HNO₂-derived intermediates are responsible. Collectively, our results indicate that the acidic pH approximately 6.5 of the CF airway mucus promotes the generation of NO₂⁻ derivative(s) that selectively kill *mucA* mutant *P. aeruginosa*. Undoubtedly, these derivatives originate from nitrous acid (HNO₂, pK_a = 3.3, where pK_a is the negative logarithm of the equilibrium constant K_a), whose equilibrium concentration increases with medium acidity. We tested this conjecture by exposing strain FRD1 to 2 different culture conditions with identical HNO₂ concentrations of approxi-

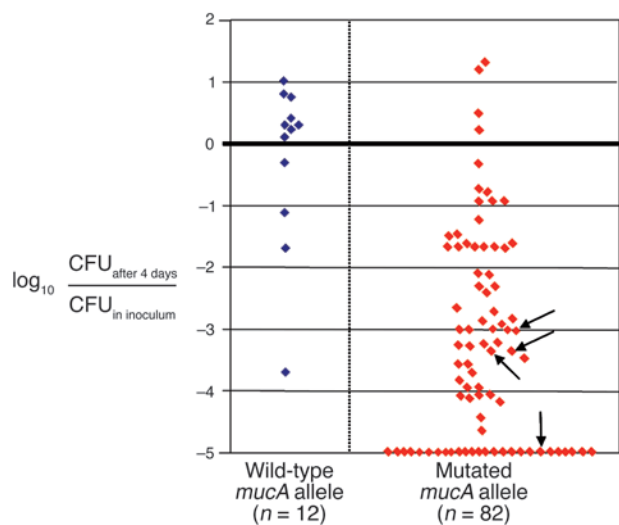


Figure 3

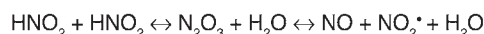
HNO₂ sensitivity of 94 different mucoid CF clinical isolates of *P. aeruginosa*. Aerobic starter cultures of each strain were diluted 100-fold in LB (pH 6.5) supplemented with 15 mM NO₂⁻. The CFU in the inoculum versus that after a 4-day anaerobic incubation were determined. The values for log₁₀ [CFU_{after 4 days}/CFU_{inoculum}] were calculated and are plotted as the viability index. The *mucA* gene of each isolate was sequenced, and mucoid strains with wild-type *mucA* alleles and those harboring *mucA* mutation were shown at left and right, respectively. Arrows indicate clinical isolates that were found to be highly resistant to amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, gentamycin, imipenem, and tobramycin.

mately 10 μM (pH 6.5, 15 mM NO₂⁻, and pH 5.5, 1.5 mM NO₂⁻). Under both conditions, equal killing of bacteria was observed, supporting the notion that formation of HNO₂ is a prerequisite for killing mucoid *P. aeruginosa* (Figure 4A). Both mucoid FRD1 and nonmucoid FRD1/*pmucA* maintained viability at these pH values when no NO₂⁻ was added (Figure 4A, left side of each plate).

At such low concentrations, HNO₂ is unlikely to directly inflict lethal lesions because it is relatively unreactive and would revert to NO₂⁻ upon penetrating bacterial membrane and entering the neutral cytoplasm. However, HNO₂ is unstable toward disproportionation that slowly generates NO and NO₃⁻. Consistent with this chemistry, our results revealed that NO accumulated upon addition of NO₂⁻ to Luria-Bertani broth (LB) at pH 6.5 (Figure 4B). After a rapid rise, NO levels plateau at approximately 490 nM, which corresponds to the decomposition of a small fraction of added NO₂⁻ and indicates that its decomposition is impeded by accumulating NO. The addition of the stoichiometric NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (carboxy-PTIO), temporarily

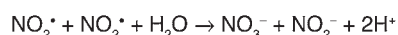
depleted NO, but the NO level was promptly restored following scavenger consumption. This dynamic behavior directly bears on the bactericidal action of acidified NO₂⁻ and is explicable by the well-established 2-step HNO₂ decomposition mechanism (Supplemental Table 2). In the first reversible step, a pair of NO and NO₂^{*} radicals are generated (Equation 1), but this reaction is so strongly shifted to the left that only 3 nM each of NO and NO₂^{*} are present at equilibrium.

Equation 1



However, the second dimerization/hydrolysis step (Equation 2) removes NO₂^{*}, thus shifting the equilibrium of Equation 1 to the right and leading to accumulation of NO to a level at which the reverse NO + NO₂^{*} reaction in Equation 1 outcompetes the NO₂^{*} dimerization/hydrolysis, making further NO accumulation extremely slow.

Equation 2



This chemistry is amenable to a kinetic analysis by computer simulation (ref. 28 and Supplemental Table 2) revealing that the initial rate of NO accumulation of approximately 4 μM/h decreases by more than 3 orders of magnitude when NO reaches approximately 500 nM; notably, identical NO accumulation profiles are predicted for 15 mM NO₂⁻, pH 6.5 and 1.5 mM NO₂⁻, pH 5.5, thereby explaining the results in Figure 4A. As observed in Figure 4B, the simulations predict 90% NO depletion in 25 minutes upon the addition of 200 nM carboxy-PTIO and clearly show that out of the 3 intermediates of NO₂⁻ decomposition (HNO₂, NO₂^{*}, and NO), only NO reacts with carboxy-PTIO. If

Table 1

Age-dependent emergence of *mucA* mutant *P. aeruginosa* from 5 different CF patients and sensitivity of each isolate to acidified NO₂⁻

Patient	Age (yr)	Mucoid	<i>mucA</i> mutation	Viability index	Note
A	2.8	NM	No	0.72	First <i>P. aeruginosa</i> colonization
	4.5	NM	No	0.13	
	6.0	M	Yes, stop at 441	-3.1	First mucoid <i>P. aeruginosa</i> isolation
B	1.2	NM	No	0.21	First <i>P. aeruginosa</i> colonization
	3.0	NM	No	1.07	
	5.3	M	No	-3.14	First mucoid <i>P. aeruginosa</i> isolation
C	5.5	M	Yes, stop at 351	-2.93	Mucoid <i>P. aeruginosa</i> isolation
	0.5	NM	No	1.3	First <i>P. aeruginosa</i> colonization
	2.5	NM	No	0.15	
D	2.9	M	Yes, stop at 441	-1.9	First mucoid <i>P. aeruginosa</i> isolation
	6.0	NM	No	0.13	First <i>P. aeruginosa</i> colonization
	7.6	NM	No	1.13	
E	10.7	M	Yes, stop at 441	-2.88	First mucoid <i>P. aeruginosa</i> isolation
	2.2	NM	No	1.2	First <i>P. aeruginosa</i> colonization
	5.3	NM	No	1.09	
	5.7	M	No	0.9	First mucoid <i>P. aeruginosa</i> isolation

The *mucA* genes of 3–4 sequential isolates from each patient were sequenced and viability index of all isolates were determined as described in Figure 3. Age provided is the age at which bacterial samples were isolated. NM, nonmucoid; M, mucoid.



Table 2
Correlation between *mucA* mutations and NO₂⁻ sensitivity

Strain	Mucoid	<i>mucA</i> mutation	Viability index	LD ₅₀
PDO300 (PAO1 <i>mucA22</i>)	M	Yes	-2.4	4.1 (± 0.2)
PAO1 <i>mucB::Tc^r</i>	M	No	0.45	N/A
PAO1 <i>mucD::Tc^r</i>	M	No	0.39	N/A
PAO1 <i>algW::Tc^r</i>	M	No	0.57	N/A
FRD1	NM	Yes	-3.84	2.8 (± 0.18)
FRD1 <i>algD::Tn501</i>	NM	Yes	<-3.48	2.5 (± 0.1)
FRD1 <i>algT(U)::Tn501</i>	NM	Yes	-3.95	2.45 (± 0.15)
Clinical isolate 35	NM	Yes	<-3.71	3.1 (± 0.08)
Clinical isolate 37	NM	Yes	<-4.12	2.2 (± 0.1)
Clinical isolate 38	NM	Yes	<-4.54	2.55 (± 0.34)
Clinical isolate 40	NM	Yes	<-3.87	2.6 (± 0.2)

FRD1 and *P. aeruginosa* clinical isolates 35, 37, 38, and 40 are nonmucoid (NM) revertants from the original mucoid (M) isolates (see Supplemental Table 1). Viability index was calculated as described in Figure 3. The LD₅₀ of NO₂⁻ was determined from a graph correlating the percent survival of strains after 24 hours of exposure to acidified NO₂⁻ ranging from 1–5 mM (see Figure 2E).

HNO₂ reacted, the addition of carboxy-PTIO would cause no change in NO concentration. If NO₂^{*} were reactive, we would observe a sharp 200-nM increase in the NO concentration upon carboxy-PTIO addition. Thus, carboxy-PTIO is a selective NO scavenger in our system.

This analysis naturally suggests that NO is involved in the microbicidal action of HNO₂. It also allows an experimentally verifiable prediction that carboxy-PTIO should have a strong protective effect when added at millimolar concentrations. As shown in Figure 4C, such protection is indeed observed. The computer modeling analysis shows that, under the conditions in Figure 4C, carboxy-PTIO will persist in solution for several weeks, keeping NO concentrations at picomolar levels. Similarly protective effects were observed with another NO scavenger, deoxyhemoglobin (Figure 4C and ref. 29). These experiments therefore establish that NO was at least potentially 1 direct or indirect contributor to the bactericidal activity of acidified NO₂⁻. This conclusion is strongly supported by the observation that strain FRD1, but not FRD1/*pmucA*, was killed when an NO/argon gas mixture was bubbled into the bacterial suspension (Figure 4D); a 380-nM solution concentration of NO maintained in this experiment was comparable to that generated by 15 mM NO₂⁻ at pH 6.5 (Figure 4B). In contrast, both strains maintained viability when treated with argon gas.

Although a link between NO depletion and the decrease of bactericidal activity of acidified NO₂⁻ clearly exists, it is premature to conclude that NO is per se the toxic agent in this system or the only toxic agent. NO depletion, whether through the action of exogenous carboxy-PTIO or endogenous NOR activity, may inhibit several downstream reactions by which HNO₂ may kill mucoid *P. aeruginosa*. These reactions alter the reaction dynamics modeled in buffer alone and may ultimately prove relevant to the observed bactericidal effect of HNO₂. For example, high NO levels promote formation of a strong nitrosating intermediate, N₂O₃ in Equation 1, which can cause “nitrosative stress” by modifying the function of proteins (30). Additionally, NO can react with iron and sulfur species in bacteria to form bioactive *S*-nitrosothiols (31) that may be potent in killing mucoid *P. aeruginosa*, an intriguing possibility given that *S*-nitrosothiol levels are low in the CF airway.

Although we strived for strictly anaerobic conditions, even remotely low levels of O₂ could be problematic because of the attendant generation of O₂⁻, which combines with NO to form peroxynitrite (ONOO⁻), an extremely bactericidal species (32, 33). We used a *sodAsodB* mutant, devoid of iron- and manganese-cofactored superoxide dismutase. As such, it is extremely sensitive to even endogenously generated O₂⁻ (34). Were trace oxygen to be reduced to O₂⁻ in the *sodAsodB* mutant in the presence of NO, then ONOO⁻ would form and kill the *sodAsodB* mutant much more rapidly than was observed in wild-type organisms. Figure 4E shows that the *sodAsodB* mutant of strain PAO1 was not sensitive to HNO₂ under anaerobic conditions. In contrast and as expected, under aerobic conditions, the *sodAsodB* mutant was more sensitive to HNO₂ (Figure 4E, compare lines shown with diamonds). These results indicate that ONOO⁻ is not formed in our anaerobic cultures and that bacterial killing is not due to this species.

Mucoid mucA mutant bacteria harbor low anaerobic NOR and NIR activity. Our next goal was to define the molecular basis of HNO₂ sensitivity of mucoid *mucA* mutant bacteria. We first measured the activity of enzymes involved in *P. aeruginosa* anaerobic respiration, including NO₃⁻ reductase (NAR), NIR, and NOR (Figure 5A). Strain FRD1 possessed approximately 3.7-fold greater NAR activity compared with nonmucoid FRD1/*pmucA* and PAO1. However, strain FRD1 possessed only 4% and approximately 20% the NIR and NOR activity, respectively, of strain PAO1 (Figure 5A). Interestingly, FRD1/*pmucA* possessed approximately 2-fold higher NO consuming activity compared with strain PAO1 (Figure 5A), suggesting a positive correlation of NOR activity with the presence of multiple copies of wild-type *MucA*. The reduced NOR activity in strain FRD1 explains in part the very limited capacity for removal of NO by this organism and, hence, its greater sensitivity to purified NO. The lack of NIR activity in strain FRD1 explained the failure of NO₂⁻ to support anaerobic growth of this strain. In addition, the low NIR activity in strain FRD1 led to the constancy of HNO₂ levels in the culture medium and the attendant increase in NO levels compared with strains that metabolize NO₂⁻. Strikingly, there was no significant loss of NO₂⁻ from the culture medium after 4 days at pH 6.5, suggesting that there was no biological reduction of NO₂⁻ in strain FRD1 and 16 different mucoid, *mucA* mutant CF isolates assessed for NO₂⁻ sensitivity (Supplemental Table 1) and remaining NO₂⁻ levels (Figure 5B). Moreover, these data also demonstrate that NO formed from HNO₂ at this pH and NO₂⁻ concentration in the presence of *mucA* mutant organisms was virtually all recycled to NO₂⁻, supporting a mechanism for NIR-deficient mutant bacteria killing that involves NO oxidation and formation of NO⁺ equivalents. These data confirm the lack of respiratory NIR activity in mucoid, *mucA* mutant *P. aeruginosa* (Figure 5B). In contrast, supernatants of wild-type strain PAO1 and FRD1/*pmucA* had little or no media NO₂⁻ remaining (Figure 5B).

Finally, because NOR activity was significantly reduced in mucoid bacteria, we postulated that mucoid organisms completely devoid of NOR activity would be more sensitive to HNO₂. Figure 5C demonstrates that a *norCB* mutant of strain FRD1 was approximately 10-fold more sensitive to acidified NO₂⁻ than were wild-type organisms.

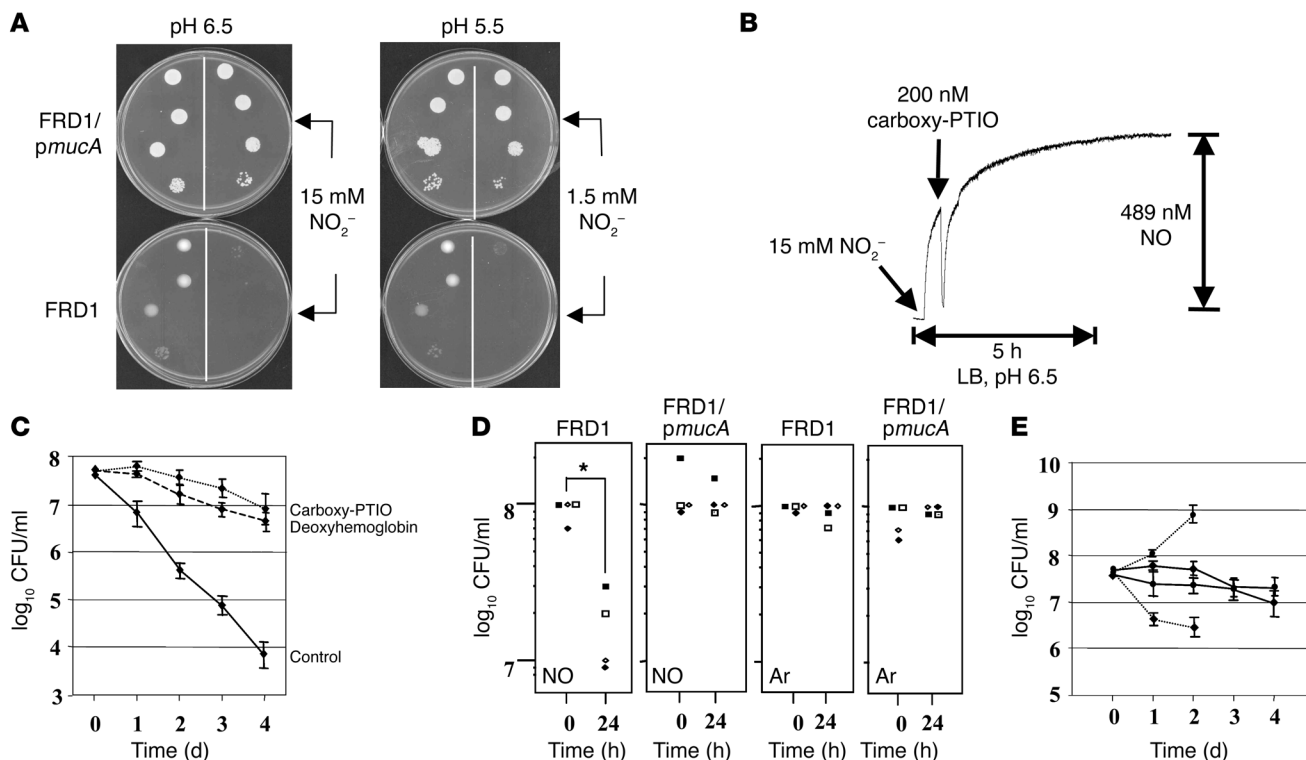


Figure 4

NO is involved in killing of mucoid *pmucA* mutant bacteria. **(A)** Anaerobic sensitivity of *P. aeruginosa* under conditions allowing for the formation of identical NO levels. Bacteria were incubated anaerobically for 4 days with or without 15 mM and 1.5 mM NO_2^- at pH 6.5 and 5.5, respectively. To enumerate viable cells, 10 μl of serial dilutions were spotted on LB agar plates and incubated at 37°C for 15 hours. The left and right side of each plate represent control and NO_2^- -exposed bacteria. **(B)** NO levels generated by 15 mM NO_2^- (pH 6.5) over time. Carboxy-PTIO (200 nM) was added, and the stoichiometric decrease in NO signal is shown. The coefficient of determination (r^2) between NO concentration and electric current (pA) was 0.9997. **(C)** Effects of NO scavengers on protection of FRD1 from acidified NO_2^- . Carboxy-PTIO (5 mM) or deoxyhemoglobin (0.5 mM) was added in the initial media (LB, pH 6.5, 15 mM NO_2^-). **(D)** Toxicity of NO toward FRD1 and FRD1/*pmucA* in LB at pH 6.5. NO (333 parts per million) balanced with argon was continuously bubbled for 24 hours anaerobically and viable bacteria were subsequently enumerated. Results of 4 experiments are shown. In NO-treated FRD1, differences in CFU between 2 time points were statistically significant (* $P < 0.01$). **(E)** *P. aeruginosa* PAO1 (circles) and isogenic *sodAsodB* double mutants (diamonds) were grown in LB, pH 6.5, containing 15 mM NO_2^- under aerobic (2 days, dotted lines) and anaerobic (4 days, solid lines) conditions. CFUs were measured daily and plotted in logarithmic scale. For aerobic samples, strains were grown with vigorous shaking (300 rpm).

HNO₂ kills mucoid P. aeruginosa in in vitro anaerobic biofilms and fresh sputum isolates from CF patients. To explore the potential clinical application of HNO_2 in the treatment of mucoid *P. aeruginosa* infections in chronic CF patients, we first tested the effect of NO_2^- on the viability of biofilm bacteria. Anaerobic biofilms of strains FRD1 and FRD1/*pmucA* were grown for 1 day in media containing NO_3^- , which supports anaerobic respiration. Since mucoid strain FRD1 lacks a flagellum (35), a surface appendage that is critical for *P. aeruginosa* biofilm initiation (36), strain FRD1 formed much weaker biofilms than those of flagellated FRD1/*pmucA* (Figure 6, compare A and B). When the FRD1 biofilm was treated with NO_2^- at pH 7.5 for 2 days, no difference in biofilm structure and cell viability was observed relative to control biofilms. In contrast, nearly complete death of biofilm organisms was observed after a 2-day incubation with NO_2^- at pH 6.5 (Figure 6A, top right). In biofilms of FRD1/*pmucA*, however, resistance to acidified NO_2^- was clearly evident (Figure 6B).

We next monitored the effect of HNO_2 on the killing of sputum isolates from CF patients. Sputum isolates CF1, -2, -3, and -4 harbored nearly 100% mucoid *pmucA* mutant bacteria, while the

CF5 isolate harbored only nonmucoid MucA-proficient *P. aeruginosa* (Figure 6C). To minimize loss of the properties acquired in vivo, these isolates were passed only once in L-broth and immediately assayed for anaerobic NO_2^- sensitivity. When these isolates were exposed to 15 mM NO_2^- at pH 6.5 under anaerobic conditions for 1 day, a sharp decrease in viability was observed in the mucoid *pmucA* mutant isolates. Again, the inherent utility of these isolates was that they were freshly isolated from CF patients and were not from long-term frozen stocks derived from CF patients. These results clearly indicate that high levels of HNO_2 selectively kill mucoid *pmucA* mutant *P. aeruginosa*.

HNO₂ kills mucoid P. aeruginosa in a sterile ultrasupernatant derived from explanted CF lungs and in mouse airways. Next, we determined whether mucoid strain FRD1 could be killed by HNO_2 in sterile ultrasupernatants (pH 6.24) of CF airway secretions derived from explanted CF lungs. This reagent arguably represents the best medium to investigate *P. aeruginosa* in the context of bacterial growth and the effects of HNO_2 ex vivo. Figure 7A shows that mucoid bacteria were actually killed faster by HNO_2 in the CF ultrasupernatants than in L-broth (Figure 2B).

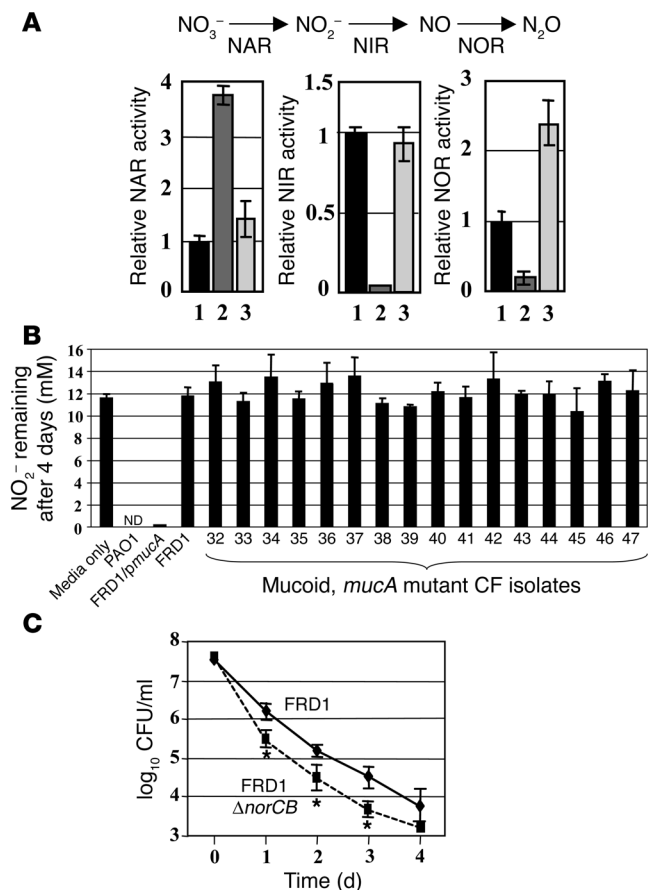


Figure 5

Significantly reduced NIR and NOR activities in *mucA* mutant *P. aeruginosa* account for the sensitivity to HNO₂. **(A)** Relative activities of NAR, NIR, and NOR in FRD1 (lane 2) and FRD1/*pmucA* (lane 3), normalized to that of the well-characterized laboratory strain PAO1 (lane 1). Each assay was performed in triplicate, and the mean ± SEM is presented. A schematic diagram of the *P. aeruginosa* anaerobic respiration pathway is shown. Bacteria were grown in LB (pH 6.5) containing 100 mM NO₃⁻ for 15 hours under anaerobic conditions. **(B)** *P. aeruginosa* strains were incubated anaerobically in LB containing 15 mM NO₂⁻, pH 6.5. After 4 days at 37°C, NO₂⁻ levels were measured in triplicate. All other *mucA* mutant clinical isolates were among those described in Supplemental Table 1. For the media-only control, no bacteria were used. ND, not detected. **(C)** Anaerobic sensitivity of FRD1 and FRD1 *norCB* mutant exposed to NO₂⁻ at pH 6.5. Experimental conditions were identical to those used in Figure 2B. Δ, deletion of a specific gene. **P* < 0.01 versus FRD1.

shows that there was no bacteria detected in mice treated for 16 days with HNO₂, while buffer control mice still harbored nearly 10⁴ mucoid organisms per lung.

To address the possibility that acidified HNO₂ can kill mucoid *mucA* mutant bacteria in the presence of nonmucoid bacteria in vivo (similar to the results obtained in vitro in Figure 2D), competitive index (CI) experiments were performed. Figure 7E demonstrates that the CI was only approximately 0.2 for mucoid, *mucA* mutant strain FRD1 relative to its complemented strain, FRD1/*pmucA*, which was approximately 1.0.

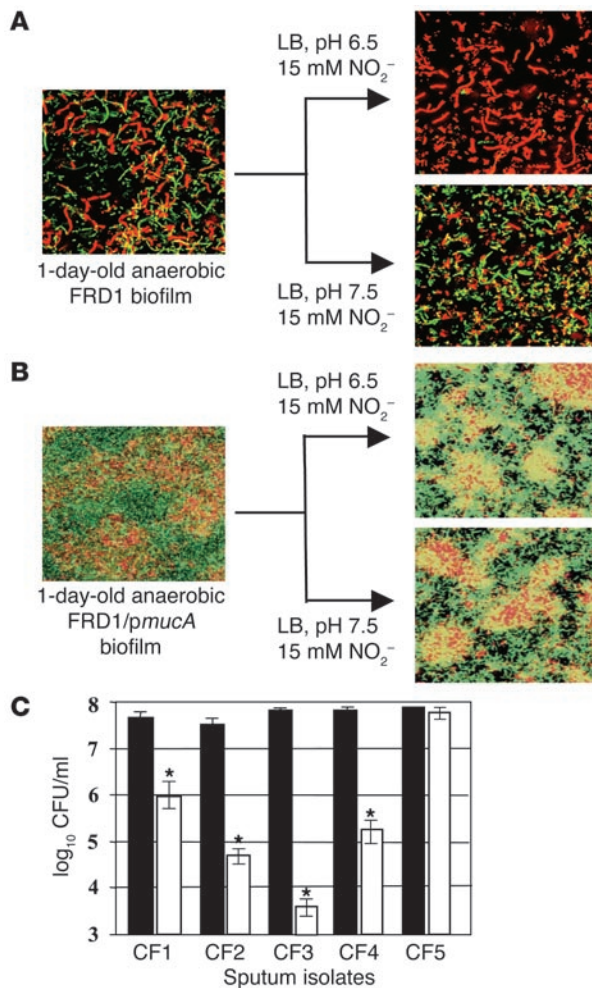
NO₂⁻ does not elicit adverse effects on airway epithelia in vitro. The clinical utility of NO₂⁻ as a treatment would be diminished if it exerted significant toxic or adverse effects on airway epithelia. Therefore, we tested the effect of NO₂⁻ on cell viability and function of cultured airway epithelia. Furthermore, since NO₂⁻ may elicit a proinflammatory response that would be undesirable in the CF airways, and NO has been reported to increase IL-8 gene transcription in a lung epithelial cell line (38), we also tested whether NO₂⁻ induces IL-8 release from cultured airway epithelia. Aerosolization, a potential therapeutic delivery route for NO₂⁻ to the CF airways, would deliver it in small volumes on the epithelial surface. To mimic this situation in vitro, we added a low volume (2 μl) of test solution containing various concentrations of NO₂⁻ to the apical surface of CF airway epithelia at pH 6.5. Exposure to concentrations as high as 20 times the dose required to kill mucoid *mucA* mutant *P. aeruginosa* exerted no cytotoxicity toward CF airway epithelia after 24 hours, as determined by lactate dehydrogenase release (Figure 8A). We performed such experiments because aerosol exposure of any effective agent, regardless of treatment, requires that significantly higher concentrations (about 25-fold) of stock solution be used so that appropriate doses are administered efficiently to the areas of interest.

In CF culture preparations mounted in Ussing chambers, basal transepithelial short circuit current (I_{sc}) was not affected by NO₂⁻ exposure (Figure 8B). Further, 15 mM NO₂⁻ failed to affect amiloride-sensitive I_{sc} (control, 15.4 ± 1.4 μA/cm²; treated, 15.7 ± 1.7 μA/cm²; *P* = 0.92) and UTP-activated I_{sc} (control, 17.7 ± 3.0 μA/cm²; treated, 18.1 ± 3.3 μA/cm²; *P* = 0.92). Consistent with these data, 15 mM NO₂⁻ did not alter transepithelial water flux (J_w) in the same cultures (Figure 8C) or trigger IL-8 release over 24 hours in CF epithelia (Figure 8D). Finally, we performed preliminary studies of the durability of NO₂⁻ on CF

Next, we measured NO levels generated from HNO₂ disproportionation in ASL collected from primary CF airway epithelia. Figure 7B indicates that NO was produced in CF ASL at levels even greater (562 nM) than those generated in LB at pH 6.5 (489 nM, Figure 4B). The higher levels of NO produced upon addition of NO₂⁻ in this milieu were likely due to the lower pH of the sample (6.43 versus 6.5).

We then determined the efficacy of HNO₂ to kill strain FRD1 in a *P. aeruginosa* chronic lung infection model. Currently, there is no animal model for the anaerobic biofilm mode of CF airway disease or a CF animal that acquires spontaneous *P. aeruginosa* infections. However, CD1 mice inoculated with agarose beads impregnated with bacteria have been useful for studying chronic lung infection by *P. aeruginosa* (37). Consistent with our in vitro results, mucoid FRD1, but not nonmucoid FRD1/*pmucA*, were decreased more than 2 logs at pH 6.5 and more than 3 logs at pH 5.5 by HNO₂⁻ in vivo (Figure 7C). Because NO concentrations derived from acidified NO₂⁻ are 10-fold greater with a reduction of 1 pH unit, our results are consistent with classical NO₂⁻ reduction chemistry. Furthermore, organisms that were recovered from the mouse airways after NO₂⁻ exposure were still sensitive to NO₂⁻ in vitro (data not shown), although these results do not clearly indicate that NO itself within the bacterial cytoplasm is the toxic species.

We also addressed whether long-term treatment with HNO₂ produced progressively decreasing airway titers of mucoid, *mucA* mutant bacteria. NO₂⁻ was instilled on a daily basis in mice infected with mucoid organisms for a period of 16 days. Figure 7D

**Figure 6**

Effect of HNO₂ on killing *P. aeruginosa* in biofilms and fresh sputum isolates. **(A)** Confocal laser microscopic analysis of anaerobic FRD1 biofilms. Live cells are stained with syto-9 (green), and dead cells are stained with propidium iodide (red). Top (x-y plane) views are projected from a stack of 125 images taken at 0.4- μ m intervals for a total of 50 μ m. Before staining, 1-day-old anaerobic FRD1 biofilms were treated anaerobically with 15 mM NO₂⁻ at pH 6.5 and 7.5 for 2 days. **(B)** Biofilms were grown as in **A** using FRD1/pmucA. **(C)** Toxicity of 15 mM NO₂⁻ (pH 6.5) toward *P. aeruginosa* sputum isolates. Viable cells in the initial inoculum (black bars) and after a 24-hour anaerobic incubation (white bars) are presented in logarithmic scale. CF1–CF4 are mucoid *mucA* mutants, and CF5 is nonmucoid and possesses a wild-type *mucA* gene. Each strain was tested in triplicate and the mean \pm SEM is presented. **P* < 0.05.

airway surfaces. We found that the half life of NO₂⁻ was approximately 5 hours (Figure 8E), indicating that NO₂⁻ is not immediately removed from the luminal side of CF airway epithelia.

Discussion

A hallmark of CF airway disease is the emergence of alginate-over-producing *P. aeruginosa*, bacteria that have genotypically and/or phenotypically changed from a nonmucoid to mucoid forms and are highly resistant to host defenses (8) and antimicrobial therapy (7). Clinically, the appearance of this organism is correlated with a marked reduction in lung function and nutritional status (39). One major mechanism for *P. aeruginosa* mucoid conversion in CF isolates involves mutations in the *mucA* gene (9). The only other known genes that are involved in mucoid conversion include *mucB* (*algN*) (27), *mucD* (25), and *algW* (25), but these mutations are not common in clinical isolates.

In this study, we believe that we have discovered the Achilles' heel of the formidable mucoid form of *P. aeruginosa*, which could lead to improved treatment for CF airway disease. Under conditions that mimicked the CF airway mucus, HNO₂ was transformed into toxic species that specifically and negatively affected viability of mucoid *P. aeruginosa*. Strikingly, *mucA* mutant bacteria were very sensitive to species derived from HNO₂, which was due at least in part to the inherently low NIR and NOR activ-

ity in these strains. In fact, recent work by Firoved et al. (40) has shown that *mucA* mutant bacteria have a markedly reduced capacity to remove the NO generated even aerobically from *S*-nitrosoglutathione. This is consistent with our results, which indicated that NO levels accumulate and remain for greater than 24 hours. Since MucA is an inner membrane-spanning protein and catalytic activities of NIR and NOR reside in the periplasmic space, we speculate that the periplasmic portion of MucA plays a crucial role in orchestrating the biological function of these periplasmic enzymes; indeed, virtually all of the critical mutations in the *mucA* gene predict defects in the periplasmic localization of MucA. Studies to define a structural relationship between MucA and potential periplasmic proteins are currently underway. HNO₂ derivatives can damage DNA (41) and modify protein micromoiety including Fe-S clusters (42), tyrosine residues (43), heme (44), and sulfhydryl groups (45). These mechanisms may be involved in the adverse effects on the overall biology of mucoid, *mucA* mutant organisms.

Our *mucA* sequencing data from 94 different strains from 5 CF clinics confirm the previous findings of Martin et al. (9) that mucoid conversion is mainly caused (87% frequency) by *mucA* mutations (Supplemental Table 1). However, variations exist in the mechanisms by which *P. aeruginosa* undergoes mucoid conversion (9, 12), and mucoid organisms that have intact *mucA* alleles are being detected, particularly in Europe. The data presented in this study indicate that only *mucA* mutant bacteria are susceptible to HNO₂.

Airway epithelial expression of iNOS does not differ between young CF patients and normal children (23). However, as CF patients age, expression of iNOS is significantly reduced in CF patients (23). This reduced expression of iNOS in chronic CF is associated with the emergence of mucoid *mucA* mutant subpopulations. Currently, however, it is unclear whether conversion to the mucoid form, which has limited capacity for NO removal, is facilitated by the abnormally low NO or *S*-nitrosothiol levels in the airways of older CF subjects (15).

Our data suggest that 15 mM NO₂⁻ kills *mucA* mutant *P. aeruginosa* in CF airways at pH 6.5. The NO chemistry of bacteria and the CF airways is complex, and several downstream mechanisms could account for the effect of HNO₂ on mucoid *P. aeruginosa*. Our data suggest that NO itself, whether directly or as a precursor to iron-nitrosyl species, may be involved in the antimicrobial effect. Of note, there is evidence that airway acid stress characterizes a variety of pulmonary disorders (46), and our

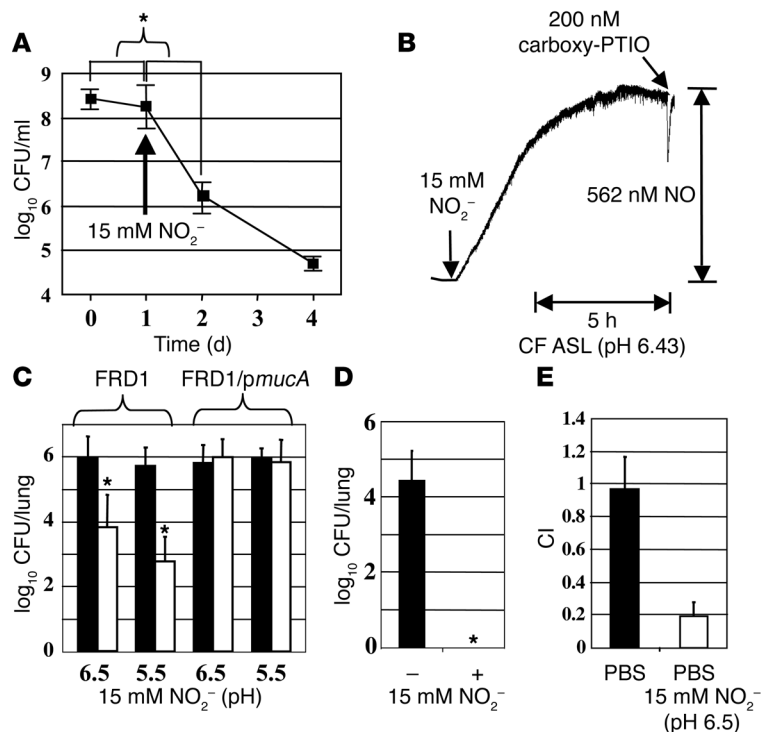


Figure 7

Applications of HNO₂-mediated killing of mucoid *P. aeruginosa* to clinical specimens. (A) Killing of FRD1 by NO₂⁻ in sterile ultrasupernatants of CF airway secretions derived from explanted CF lungs. Bacteria were incubated anaerobically for 24 hours, and 15 mM NO₂⁻ was added. CFUs were determined (*n* = 3) and plotted as the X ± SEM versus time. **P* < 0.01 versus CFU decrease before adding NO₂⁻. (B) NO generation in CF ASL by 15 mM NO₂⁻. Except for the media and pH, experimental conditions were identical to those used in Figure 4B. (C) Effects of NO₂⁻ on killing of FRD1 and FRD1/*pmucA* in mouse lungs. CD1 mice were infected with FRD1 or FRD1/*pmucA* as described in Methods. Infected mice were treated with buffer (black bars) and buffered NO₂⁻ (white bars) daily, and viable bacteria from the lung homogenates were enumerated. *n* = 8 per group. **P* < 0.01 versus buffer alone. (D) Effects of long-term NO₂⁻ treatment on killing of FRD1 in mouse lungs. Another group of FRD1-infected mice were treated daily with buffer (-, 50 mM sodium phosphate, pH 6.5) or buffer with 15 mM NO₂⁻ (+) for 16 days (*n* = 8 per group). Organisms surviving treatment with buffer and NO₂⁻ were shown in logarithmic scale. **P* < 0.01 versus buffer alone. (E) CI experiments with 10⁶ FRD1 and FRD1/*pmucA* intratracheally instilled into CD1 mouse airways and incubated for 6 days prior to harvesting of mouse lungs and enumeration of CFUs after homogenization.

results are consistent with those of Hunt et al. (47), which suggest that delivery of concentrated NO₂⁻ to airway regions with low pH likely serves to generate NO and *S*-nitrosothiols. Indeed, NO generated in animal studies of inhaled NO₂⁻ may arise from HNO₂ formation (18). Inhaled NO₂⁻ has appeal as a CF therapy because it may (a) provide sustained NO release, (b) exploit the low pH of the CF airway epithelial mucus layer, (c) selectively inhibit growth of mucoid, *muca* mutant *P. aeruginosa* when protonated to form HNO₂, and (d) provide a chemical feedback mechanism that maintains the desired NO levels in response to its consumption or removal (Figure 4B). Therefore, we hope that the data provided in this study stimulates further investigation. However, it is important to caution that (a) the airway pH may not be homogeneous in vivo as excessive regional acidity and high HNO₂ levels could result in airway injury; (b) excessive NO production in the airway could inhibit platelet aggregation, potentially aggravating hemoptysis; (c) HNO₂ produces carcinogenic nitrosoamines; (d) the nitrogen redox chemistry in the CF airway in vivo is exceptionally complex, and several unexpected (and untoward) reactions could result from formation of high airway levels of HNO₂; (e) for denitrifying organisms that have intact NIR and NOR activity (including the *Aspergillus* species), inhaled NO₂⁻ could paradoxically promote growth as a nutrient; (f) there are no human data regarding the safety of inhaling near-molar quantities of NO₂⁻, which could have adverse local and systemic effects; and (g) the precise mechanism by which HNO₂ affects mucoid *P. aeruginosa* growth is not known. Still, despite these known pitfalls, mucoid *P. aeruginosa* organisms were previously considered impossible to eradicate from the airways of patients with chronic CF lung disease. We believe that our data offer hope that effective treatment strategies can be designed with the ultimate goal of eradicating this formidable foe in CF lung disease.

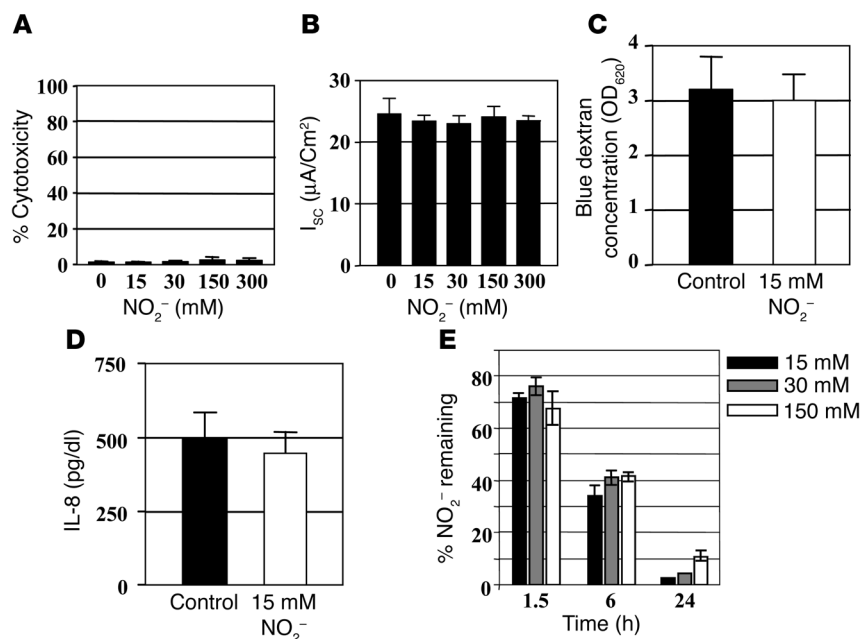
Methods

Bacterial culture and enzymatic assays. *P. aeruginosa* strains used in this study included nonmucoid strain PAO1 (48), CF isolate FRD1 (49), and sputum isolates from CF patients at 5 different North American clinics, totaling 94 strains (Supplemental Table 1). All procedures using human patients were approved by the respective university's Institutional Review Board with respect to informed consent issues. Complementation of FRD1 *muca* was achieved by transformation with the plasmid *ptacmuca* (9). Aerobic starter cultures were grown in LB (10 g tryptone, 5 g NaCl, and 5 g yeast extract per liter) at 37°C. Anaerobic growth was achieved in a Coy anaerobic chamber (Coy Laboratory Products). To support anaerobic respiration, KNO₃ and/or NaNO₂ (Sigma-Aldrich) were added to the medium. The pH of the medium was adjusted with 100 mM sodium phosphate (for pH 6.5) or 100 mM sodium acetate (for pH 5.5). To enumerate viable bacteria, serial dilutions were spotted on LB agar plates. NO₂⁻ sensitivity using a filter paper disk assay was performed as previously described (31). NAR, NIR, and NOR activities were measured as described previously (21). Biofilm staining and image acquisition were accomplished as described previously (21).

NO experiments. NO levels were measured polarimetrically using a NO electrode system (Model Apollo 400; World Precision Instruments Inc.) according to the manufacturer's instructions. The NO gas exposure study was performed as described previously (32).

Measurement of pH in airway secretions. In situ pH measurements of mucopurulent airway secretions from CF airways were made by inserting the tip of a pH microelectrode (MI-413; Microelectrodes Inc.) into mucopurulent secretions within lobar, segmental, and subsegmental bronchi of freshly explanted lungs from 9 CF patients. Lungs were removed at the time of organ transplantation. Duplicate readings at 3 different sites per patient were recorded, and the mean value from each measurement was used for analysis.

Infection of mouse airways. CD1 mice (*n* = 8) were infected with approximately 10⁶ FRD1 or FRD1/*pmucA* entrapped in agar beads intratracheally as previously described (33). Following 24 hours' incubation, mouse lungs were instilled with 25 µl of 15 mM NO₂⁻ at pH 5.5 (in 0.1 M acetate buffer) or pH 6.5

**Figure 8**

Effect of NO_2^- on function of cultured human airway epithelia. (A) Culture preparations of human CF airway epithelium (duplicate preparations, $n = 3$) were exposed apically to 2 μl of varying concentrations of NO_2^- . After 24 hours, lactate dehydrogenase activity in the basolateral media was measured to monitor cytotoxicity. (B) CF airway epithelial cultures were mounted in Ussing chambers and treated with 2 μl of liquid containing NO_2^- at varying concentrations. Transepithelial short circuit current (I_{sc} , in $\mu\text{A}/\text{cm}^2$) was measured to monitor change in bioelectric properties. (C) CF airway epithelial cultures (triplicate preparations, $n = 4$), were treated luminally with 100 μl Krebs bicarbonate Ringer buffer containing 2% blue dextran and supplemented with either 15 mM NaCl (control) or NO_2^- for 24 hours. Transepithelial water flux (J_v) was calculated by measuring blue dextran concentration optically after 24 hours in microaliquots of sampled luminal liquid. (D) IL-8 release assay using primary cultures of CF airway epithelia ($n = 4$) exposed to 15 mM NO_2^- compared with control cultures. (E) Determination of NO_2^- half life on the surface of airway epithelia. The 3 different concentrations of NO_2^- were applied to airway epithelial cell monolayers, and NO_2^- levels were assayed at the indicated intervals ($n = 3$ for each measurement). $P = \text{NS}$ for all comparisons (ANOVA).

(in 0.1 M phosphate buffer) intranasally twice daily. On the fifth day mice were sacrificed, and the viable bacteria from serially diluted lung homogenates were enumerated. One group of FRD1-infected mice was treated with 15 mM NO_2^- at pH 6.5 as described above for 16 days to examine whether bacteria develop resistance after prolonged exposure to NO_2^- . All animal studies were performed in accordance with the protocols approved by the Animal Care Committee of the University of Cincinnati College of Medicine.

In vivo competition assays. In vivo competition assays between mucoid *mucA* mutant strain FRD1 and FRD1/*pmucA* embedded in agar beads were carried out by infecting CD1 mice ($n = 6$) intratracheally (2×10^5 bacteria) with a 1:1 ratio of each strain as previously described (50). Twenty-four hours after infection, mouse lungs were instilled with 25 μl of PBS at pH 6.5 or PBS containing 15 mM NaNO_2 at pH 6.5 twice daily for 4 days. Infected lungs were recovered after 6 days for bacterial load and CI calculations. The CI was defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria (51). Thus, if a mutant strain is as competitive as its isogenic wild-type parent, a value of 1 will be achieved. A CI of less than 1 implies that the mutant is not as competitive as the wild-type strain.

Biological properties of airway epithelia in response to NO_2^- . Primary bronchial epithelial cultures, grown on Transwell Col (1.13 cm^2 surface area) permeable supports (52), were mounted in Ussing chambers (ADInstruments Co.), and

bioelectric properties were analyzed as previously described (53). IL-8 concentrations in basolateral media were measured using commercially available antibody pairs (R&D Systems) according to the manufacturer's instructions. Cellular cytotoxicity was assessed by comparing release of lactate dehydrogenase into the basolateral media of cultured airway epithelial cell preparations treated apically with varying NO_2^- concentrations. Lactate dehydrogenase was measured using a commercially available spectrophotometric assay kit (BioVision Research Products). For experiments measuring transepithelial water flux, culture preparations were treated apically with 100 μl of Krebs bicarbonate Ringer solution containing 2% blue dextran (a cell-impermeable volume marker dye) supplemented with 15 mM NO_2^- . After 24 hours, microaliquots (2–5 μl) of apical liquid were collected and stored at -20°C until analyzed. Blue dextran concentrations were measured spectrophotometrically. To determine the half life of NO_2^- on the surface of cultured airway epithelia, NO_2^- levels were measured by the Griess reaction (54), and the percent rate of NO_2^- removal was calculated.

Preparation of sterile ultrasupernatants of CF airway secretions. Purulent secretions were harvested from the airways of CF lungs that were removed at the time of transplantation. Purulent secretions were centrifuged (100,000 g for 1 hour) and passed through a sterile filter (0.22 μm , Costar 8110 mStar; Millipore).

Statistics. Results are presented as mean \pm SEM. Student's t test (2-tailed, unequal variance, for Figures 1–6) and ANOVA (Figure 7) were used to analyze the significance of differences between experimental groups. A P value less than 0.05 was considered statistically significant. Kinetic modeling was carried out using the INTKIN computer program, developed at the Brookhaven National Laboratory by Harold A. Schwarz (55). The necessary rate data were obtained from the literature (56).

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