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

Sang Sun Yoon,† Ray Coakley,‡ Gee W. Lau,§ Sergei V. Lymar,* Benjamin Gaston,†,‡ Ahmet C. Karabulut,† Robert F. Hennigan,† Sung-Hei Hwang,† Garry Buettner,§ Michael J. Schurr,§ Joel E. Mortensen,∥ Jane L. Burns,∥ David Speert,∥ Richard C. Boucher,‡ and Daniel J. Hassett†

†Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA.‡Cystic Fibrosis Pulmonary Research and Treatment Center and Department of Pulmonary Biology, University of North Carolina, Chapel Hill, North Carolina, USA. §Pulmonary Biology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA.∥Department of Chemistry, Brookhaven National Laboratory, Upton, New York, USA. *Department of Cell Biology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. ¶Department of Pediatric Critical Care, University of Virginia School of Medicine, Charlottesville, Virginia, USA. ¶Pulmonary Biology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. ¶Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, New York, USA. ¶Department of Chemistry, University of Iowa, Iowa City, Iowa, USA. ¶Department of Microbiology, Tulane University, New Orleans, Louisiana, USA. ¶Department of Pediatrics, Pathology and Laboratory Medicine, Children’s Hospital, Cincinnati, Ohio, USA. ¶Infectious Diseases Section, Children’s Hospital and Regional Medical Center, Seattle, Washington, USA. ¶Department of Pediatrics, University of British Columbia, Vancouver, British Columbia, Canada.

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 persist during anaerobic exposure to 15 mM nitrite (NO$_2^-$) at pH 6.5, which mimics CF airway mucus. Killing required a pH lower than 7, implicating formation of nitrous acid (HNO$_2$) and NO, that adds NO equivalents to cellular molecules. Eighty-seven percent of CF isolates possessed *mucA* mutations and were killed by HNO$_2$ (3-log reduction in 4 days). Furthermore, antibiotic-resistant strains determined were also equally sensitive to HNO$_2$. More importantly, HNO$_2$ 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 HNO$_2$ for 16 days. HNO$_2$ at these levels of acidity and NO$_2^-$ also had no adverse effects on cultured human airway epithelia in vitro. In summary, selective killing by HNO$_2$ 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, isorotonic 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 (HCO$_3^-$) 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-consumptive activities of airway epithelium, *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-α factor (9). Without MucA, the extracytoplasmic α 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 H$_2$O$_2$ 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-tetramethylimidazoline-1-oxyl-3-oxide; CF, cystic fibrosis; Cl, competitive index; LB, Luria-Bertani broth; NAR, nitrate reductase; NIR, nitrite reductase; NOD, 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.

Citation for this article: J Clin Invest. 116:436–446 (2006). doi:10.1172/JCI24684.
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$_2^-$ 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, iNOS activity is significantly upregulated in response to proinflammatory cytokines and constitutively expressed in the airways but is inducible and upregulated in response to proinflammatory cytokines and bacterial LPS (22). 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, mucA 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/pmueA. Strain FRD1 is the best characterized mucoid, mucA mutant derived from a CF patient (24). However, no difference in viability patterns was observed (Figure 2A). Using 15 mM NO$_3^-$, however, mucoid FRD1 was killed at a rate of approximately 90% per day, while nonmucoid strains PA01 and FRD1/pmueA remained viable over the 4-day incubation (Figure 2B).

After discovering this unique NO$_3^-$ sensitivity of mucA mutant strain FRD1, we also found that NO$_3^-$ killed these bacteria more effectively at a lower pH (Figure 2C), while little or no killing was observed in strain FRD1/pmueA at pH 6.0–7.5 (Figure 2C).

To test whether NO$_3^-$ killing of mucoid bacteria occurs in the presence of nonmucoid bacteria, mucoid and nonmucoid P. aeruginosa were mixed and treated with 15 mM NO$_3^-$ anaerobically. Strain FRD1 consistently lost viability at 3 different bacterial test ratios, while nonmucoid FRD1/pmueA maintained viability (Figure 2D).

We next characterized the dose-effect relationship between NO$_3^-$ concentration and killing of mucoid bacteria. Figure 2E shows that 90–95% of the bacteria were killed by 15 mM NO$_3^-$ and the LD$_{50}$ was approximately 3 mM NO$_3^-$ after 24 hours. The remaining organisms did not develop resistance to NO$_3^-$ when we extended our study to 12 days (Figure 2F), demonstrating that all organisms were killed during this time period.

Other mucoid, mucA mutant CF isolates are also sensitive to NO$_3^-$. To test whether NO$_3^-$ sensitivity is a trait of all mucA mutant mucoid CF isolates, the mucA 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 mucA mutations, leading to either premature termination of translation (88%) or a loss of the stop codon (12%), thereby confirming previous findings (13) that mucA 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
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 mucoid isolates had a wild-type mucA allele, indicating the presence of other mechanisms or mutations allowing for mucoid conversion. Upon anaerobic treatment with 15 mM NO\textsubscript{2} at pH 6.5, almost all of the mucA mutant mucoid isolates (78 of 82 strains) showed increased susceptibility to NO\textsubscript{2}, with 74% killed by more than 2 logs relative to the mean of the nonmucoid MucA-proficient organisms (Figure 3). Importantly, 4 strains that were deemed antibiotic resistant were still sensitive to anaerobic treatment of acidified NO\textsubscript{2} (Figure 3, arrows). Out of 12, however, 8 mucoid isolates with a wild-type mucA allele were resistant to acidified NO\textsubscript{2}. These results suggest that NO\textsubscript{2} 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 mucoid P. aeruginosa as the patient aged, a hallmark of chronic infection (Table 1). As predicted, initial airway colonization of each patient was by nonmucoid P. aeruginosa. Most importantly, however, mucoid variants that were detected in patients A, B, C, and D possessed mutated mucA genes and were all killed by 15 mM NO\textsubscript{2} (1.9–3.1 logs). These results indicate that the genotypic and phenotypic switch to the mucoid form that is sensitive to NO\textsubscript{2} treatment can occur in patients less than 3 years of age (see patient C).

mucA mutations are responsible for NO\textsubscript{2} sensitivity. The above results suggest that mucA mutations could be responsible for the enhanced sensitivity to NO\textsubscript{2}. Since mucA mutant bacteria overproduce alginate, we initiated experiments to test whether NO\textsubscript{2} 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 NO\textsubscript{2} (Table 2). Two FRD1-derived nonmucoid mutants [ΔalgD, lacking GDP-mannose dehydrogenase, and AlgT(U), lacking AlgT(U)] were equally sensitive to killing by NO\textsubscript{2} (Table 2). We next tested whether NO\textsubscript{2} also killed mucB, mucD, and algW mutants of strain PAO1. Other than mucA, the aforementioned genes are the only reported loci whose intact alleles are responsible for enhanced NO\textsubscript{2} sensitivity (25–27). In contrast to mucA mutant bacteria, these mutants were not sensitive to NO\textsubscript{2} (Table 2). Most critically, 5 nonmucoid revertants of mucoid strains were still sensitive to HNO\textsubscript{2} (see Table 2). Therefore, our results suggest that NO\textsubscript{2} sensitivity is MucA- and not alginate dependent. Finally, the LD\textsubscript{30} of NO\textsubscript{2} for sensitive strains was almost identical to that for FRD1 (Figure 2E), suggesting that the rate at which these organisms were killed by NO\textsubscript{2} was similar to that of strain FRD1.

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

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-tetramethylimidazoline-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 NO₃⁻ radicals are generated (Equation 1), but this reaction is so strongly shifted to the left that only 3 nM each of NO and NO₃⁻ are present at equilibrium.

### Equation 1

\[ \text{HNO}_2 + \text{HNO}_2 \leftrightarrow \text{N}_2\text{O}_4 + \text{H}_2\text{O} \leftrightarrow \text{NO} + \text{NO}_3^- + \text{H}_2\text{O} \]

However, the second dimerization/hydrolysis step (Equation 2) removes NO₃⁻, thus shifting the equilibrium of Equation 1 to the right and leading to accumulation of NO to a level at which the reverse NO + NO₃⁻ reaction in Equation 1 outcompetes the NO₂⁻ dimerization/hydrolysis, making further NO accumulation extremely slow.

### Equation 2

\[ \text{NO}_2^- + \text{NO}_3^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + \text{NO}_2^- + 2\text{H}^+ \]

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, NO₃⁻, 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⁻

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

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.
Although we strive 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 sodA sodB 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 sodA sodB mutant in the presence of NO, then ONOO⁻ would form and kill the sodA sodB mutant much more rapidly than was observed in wild-type organisms. Figure 4E shows that the sodA sodB mutant of strain PAO1 was not sensitive to HNO₃ under anaerobic conditions. In contrast and as expected, under aerobic conditions, the sodA sodB mutant was more sensitive to HNO₃ (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 HNO₃ sensitivity of mucoid mucA mutant bacteria. We first measured the activity of enzymes involved in *P. aeruginosa* anaerobic respiration, including NOx 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 explains 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 HNO₃ 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 NO₃⁻ sensitivity (Supplemental Table 1) and remaining NO₃⁻ levels (Figure 5B).

Table 2

**Correlation between mucA mutations and NO₃⁻ sensitivity**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mucoid</th>
<th>mucA mutation</th>
<th>Viability index</th>
<th>LD₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDO300 (PAO1 mucA22) M Yes –2.4 4.1 (± 0.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1 mucB:Tc' M No 0.45 N/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1 mucD:Tc' M No 0.39 N/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1 algW:Tc' M No 0.57 N/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRD1 NM Yes –3.84 2.8 (± 0.18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRD1 algD:Tn501 NM Yes –3.48 2.5 (± 0.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRD1 algT(U):Tn501 NM Yes –3.95 2.45 (± 0.15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical isolate 35 NM Yes –3.71 3.1 (± 0.08)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical isolate 37 NM Yes –4.12 2.2 (± 0.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical isolate 38 NM Yes –4.54 2.55 (± 0.34)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical isolate 40 NM Yes –3.87 2.6 (± 0.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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).

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 sodA sodB 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 sodA sodB mutant in the presence of NO, then ONOO⁻ would form and kill the sodA sodB mutant much more rapidly than was observed in wild-type organisms. Figure 4E shows that the sodA sodB mutant of strain PAO1 was not sensitive to HNO₃ under anaerobic conditions. In contrast and as expected, under aerobic conditions, the sodA sodB mutant was more sensitive to HNO₃ (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 HNO₃ sensitivity of mucoid mucA mutant bacteria. We first measured the activity of enzymes involved in *P. aeruginosa* anaerobic respiration, including NOx 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 HNO₃ 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 NO₃⁻ sensitivity (Supplemental Table 1) and remaining NO₃⁻ levels (Figure 5B).

Moreover, these data also demonstrate that NO formed from exogenous carboxy-PTIO or endogenous NOR activity, may inhibit several downstream reactions by which HNO₃ 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 HNO₃. 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.
HNO$_2$ 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).
Next, we measured NO levels generated from HNO$_2$ 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 NO$_2^-$ in this milieu were likely due to the lower pH of the sample (6.43 versus 6.5).

We then determined the efficacy of HNO$_2$ 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 HNO$_2^-$ in vivo (Figure 7C). Because NO concentrations derived from acidified NO$_2^-$ are 10-fold greater with a reduction of 1 pH unit, our results are consistent with classical NO$_2^-$ reduction chemistry. Furthermore, organisms that were recovered from the mouse airways after NO$_2^-$ exposure were still sensitive to NO$_2^-$ 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 HNO$_2$ produced progressively decreasing airway titer of mucoid, mucA mutant bacteria. NO$_2^-$ was instilled on a daily basis in mice infected with mucoid organisms for a period of 16 days. Figure 7D shows that there was no bacteria detected in mice treated for 16 days with HNO$_2$, while buffer control mice still harbored nearly 10$^4$ mucoid organisms per lung.

To address the possibility that acidified HNO$_2$ 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.

NO$_2^-$ does not elicit adverse effects on airway epithelia in vitro. The clinical utility of NO$_2^-$ as a treatment would be diminished if it exerted significant toxic or adverse effects on airway epithelia. Therefore, we tested the effect of NO$_2^-$ on cell viability and function of cultured airway epithelia. Furthermore, since NO$_2^-$ 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 NO$_2^-$ induces IL-8 release from cultured airway epithelia. Aerosolization, a potential therapeutic delivery route for NO$_2^-$ 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 NO$_2^-$ 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 NO$_2^-$ exposure (Figure 8B). Further, 15 mM NO$_2^-$ failed to affect amiloride-sensitive I$_{sc}$ (control, 15.4 ± 1.4 μA/cm$^2$; treated, 15.7 ± 1.7 μA/cm$^2$; P = 0.92) and UTP-activated I$_{sc}$ (control, 17.7 ± 3.0 μA/cm$^2$; treated, 18.1 ± 3.3 μA/cm$^2$; P = 0.92). Consistent with these data, 15 mM NO$_2^-$ 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 NO$_2^-$ on CF
airway surfaces. We found that the half life of NO$_2$ was approximately 5 hours (Figure 8E), indicating that NO$_2$ 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, HNO$_3$ 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 HNO$_3$, which was due at least in part to the inherently low NIR and NOR activity 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. HNO$_2$ derivatives can damage DNA (41) and modify protein micromoieties 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 HNO$_3$.

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 NO$_2$ 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 HNO$_3$ 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
results are consistent with those of Hunt et al. (47), which suggest that delivery of concentrated NO\textsubscript{2} to airway regions with low pH likely serves to generate NO and S-nitrosothiols. Indeed, NO generated in animal studies of inhaled NO\textsubscript{2} may arise from HNO\textsubscript{2} formation (18). Inhaled NO\textsubscript{2} 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 \textit{P. aeruginosa} when prototoned to form HNO\textsubscript{2}, 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 HNO\textsubscript{2} levels could result in airway injury; (b) excessive NO production in the airway could inhibit platelet aggregation, potentially aggravating hemoptysis; (c) HNO\textsubscript{2} produces carcinogenic nitrosamines; (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 HNO\textsubscript{2}; (e) for denitrifying organisms that have intact NIR and NOR activity (including the \textit{Aspergillus} species), inhaled NO\textsubscript{2} could paradoxically promote growth as a nutrient; (f) there are no human data regarding the safety of inhaling near-molar quantities of NO\textsubscript{2}, which could have adverse local and systemic effects; and (g) the precise mechanism by which HNO\textsubscript{2} affects mucoid \textit{P. aeruginosa} growth is not known. Still, despite these known pitfalls, mucoid \textit{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.

\textbf{Methods}

\textit{Bacterial culture and enzymatic assays.} \textit{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 \textit{ptac} mucA (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, KNO\textsubscript{2} and/or NaNO\textsubscript{2} (Sigma-Aldrich) were added to the medium. The pH of the medium was adjusted with 100 mM sodium phosphate (for pH 6.5) or buffer with 15 mM NO\textsubscript{2} (+) for 16 days (\(n = 8\) per group). Organisms surviving treatment with buffer and NO\textsubscript{2} were shown in logarithmic scale. \(P < 0.01\) versus buffer alone.

\textit{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).

\textit{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 sterile ultraspotnats of CF airway secretions derived from explanted CF lungs. Bacteria were incubated anaerobically for 24 hours, and 15 mM NO\textsubscript{2} was added. CFUs were determined (\(n = 3\)) and plotted as the X ± SEM versus time. \(P < 0.01\) versus CFU decrease before adding NO\textsubscript{2}.

\textit{Effects of NO\textsubscript{2} generation in CF ASL by 15 mM NO\textsubscript{2}}. NO generation in CF ASL by 15 mM NO\textsubscript{2}. Except for the media and pH, experimental conditions were identical to those used in Figure 4B.

\textit{Effect of long-term NO\textsubscript{2} treatment on killing of FRD1 in mouse lungs.} Another group of FRD1-infected mice were treated daily with buffer (\(\sim 50\) mM sodium phosphate, pH 6.5) or buffer with 15 mM NO\textsubscript{2} (+) for 16 days (\(n = 8\) per group). Organisms surviving treatment with buffer and NO\textsubscript{2} were shown in logarithmic scale. \(P < 0.01\) versus buffer alone.

\textit{Cl experiments with 10\textsuperscript{6} FRD1 and FRD1/pmueC} intratracheally instilled into CD1 mouse airways and incubated for 6 days prior to harvesting of mouse lungs and enumeration of CFUs after homogenization.
The Journal of Clinical Investigation

1 implies that the mutant is not as competitive as the wild-type strain. A CI of less than 1 will be achieved. The lack of competition for airway epithelia suggests the mutant strain FRD1 and FRD1/pucA 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 liquid containing NO₂⁻ at varying concentrations. The 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 NO₂⁻ at pH 6.5 as described above for 16 days to examine whether bacteria develop resistance after prolonged exposure to NO₂⁻. All animal experiments 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/pucA 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 NaNO₂ 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 indicates that the mutant is not as competitive as the wild-type strain.

**Biological properties of airway epithelia in response to NO₂⁻.** Primary bronchial epithelial cell cultures, grown on Transwell Col (1.13 cm² surface area) permeable supports (52), were mounted in Ussing chambers (ADIInstruments 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 NO₂⁻ 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 NO₂⁻. 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 NO₂⁻ on the surface of cultured airway epithelia, NO₂⁻ levels were measured by the Griess reaction (54), and the percent rate of NO₂⁻ 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 ± 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).

Acknowledgments

This work was supported by NIH Public Health Service grants (AI-40541 and GM-69845) and Cystic Fibrosis Foundation grant HASSETT03P0 to D.J. Hassett. Research at Brookhaven National Laboratory was carried out under the auspices of the US Department of Energy under contract DE-AC02-98CH10886 from the Division of Chemical Sciences, Office of Basic Energy Sciences.

Received for publication February 8, 2005, and accepted in revised form November 29, 2005.

Address correspondence to: Daniel J. Hassett, Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, Ohio 45267-0524, USA. Phone: (513) 558-1154; Fax: (513) 558-8474; E-mail: Daniel.Hassett@uc.edu.

Sang Sun Yoon’s present address is: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts, USA.