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

Sang Sun Yoon,¹ Ray Coakley,² Gee W. Lau,³ Sergei V. Lymar,⁴ Benjamin Gaston,⁵,⁶ Ahmet C. Karabulut,¹ Robert F. Hennigan,⁷ Sung-Hei Hwang,¹ Garry Buettner,⁸ Michael J. Schurr,⁹ Joel E. Mortensen,¹⁰ Jane L. Burns,¹¹ David Speert,¹² Richard C. Boucher,² and Daniel J. Hassett¹

¹Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. ²Cystic Fibrosis Pulmonary Research and Treatment Center and Department of Pulmonary Biology, University of North Carolina, Chapel Hill, North Carolina, USA. ³Pulmonary Biology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. ⁴Department of Chemistry, Brookhaven National Laboratory, Upton, New York, USA. ⁵Department of Cell Biology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. ⁶Department of Pediatric Critical Care, University of Virginia School of Medicine, Charlottesville, Virginia, USA. ⁷Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, New York, USA. ⁸Department of Chemistry, University of Iowa, Iowa City, Iowa, USA. ⁹Department of Microbiology, Tulane University, New Orleans, Louisiana, USA. ¹⁰Department of Pediatrics, Pathology and Laboratory Medicine, Children’s Hospital, Cincinnati, Ohio, USA. ¹¹Infectious Diseases Section, Children’s Hospital and Regional Medical Center, Seattle, Washington, USA. ¹²Department 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 perish during anaerobic exposure to 15 mM nitrite (NO\textsubscript{2}\textsuperscript{-}) at pH 6.5, which mimics CF airway mucus. Killing required a pH lower than 7, implicating formation of nitrous acid (HNO\textsubscript{2}) and NO\textsubscript{2}, which adds NO equivalents to cellular molecules. Eighty-seven percent of CF isolates possessed mucA mutations and were killed by HNO\textsubscript{2} (3-log reduction in 4 days). Furthermore, antibiotic-resistant strains determined were also equally sensitive to HNO\textsubscript{2}. More importantly, HNO\textsubscript{2} killed mucoid bacteria (a) in anaerobic biofilms; (b) in vitro in ultrsupernatants 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\textsubscript{2} for 16 days. HNO\textsubscript{2} at these levels of acidity and NO\textsubscript{2} also had no adverse effects on cultured human airway epithelia in vitro. In summary, selective killing by HNO\textsubscript{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\textsuperscript{-} channel, it serves to coordinate Na\textsuperscript{+} absorption and Cl\textsuperscript{-} 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\textsuperscript{+} 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\textsubscript{3}\textsuperscript{-}) ion transport (3).

Two 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-\(\alpha\)-factor (9). Without MucA, the extracellular\(\alpha\)-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\textsubscript{2}O\textsubscript{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,
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, 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 mucA mutations. We investigated the mechanism of this effect in vitro in 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, *mucA* 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, 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/pmuca. 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$_2^-$, 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 *mucA* 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$_3^-$ 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, mucA mutant CF isolates are also sensitive to NO$_2^-$.* To test whether NO$_2^-$ 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/JCI4684DS1). The most abundant mutations were
The mutant, PDO300, carrying mucA mutations could be identified from 5 different young CF patients, we consistently detected alginate overproduction. Although most important, undoubtedly, these derivatives or mutations allowing for mucoid conversion.

When primarily kill by 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 mucoid mutants [ΔalgD, lacking GDP-mannose dehydrogenase, and ΔalgT(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 mucA mutants 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{50} 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-

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Figure 2
Mucoid P. aeruginosa FRD1 is selectively killed by NO\textsubscript{2}(-) in a pH-dependent manner. Anaerobic growth of P. aeruginosa strains at pH 6.5 using 15 mM NO\textsubscript{2}(-) or NO\textsubscript{2} as a terminal electron acceptor. Aerobic overnight suspensions of PAO1, FRD1, and FRD1/pmucA were diluted 100-fold for the main anaerobic culture with NO\textsubscript{2}(-) (A) or NO\textsubscript{2} (B). CFUs were enumerated each day and plotted as linear (A) and logarithmic (B) graphs. (C) NO\textsubscript{2}(-) 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 NO\textsubscript{2}(-), the plates were incubated anaerobically for 48 hours and scanned for viewing the zone of killing. To support anaerobic growth, 15 mM NO\textsubscript{2}(-) was included in the media. (D) Mucoid strain FRD1 (gray bars) and nonmucoid FRD1/pmucA (black bars) were incubated together at the indicated ratios (FRD1/pmucA:FRD1) for 5 days in the presence of 15 mM NO\textsubscript{2}(-), pH 6.5, after which CFUs were determined. *P < 0.001 versus CFU in the initial inoculum. (E) Dose-response killing of mucoid strain FRD1 by NO\textsubscript{2}(-). Bacteria were suspended in LB (pH 6.5) with various amounts of NO\textsubscript{2}(-) for 24 hours under anaerobic conditions. Survival against NO\textsubscript{2}(-) is presented as the percentage of CFU relative to that in initial inoculum. (F) Long-term anaerobic exposure of mucoid strain FRD1 to 15 mM NO\textsubscript{2}(-) at pH 6.5. All experiments were performed in triplicate and presented as mean ± SEM.

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

Equation 1
\[
\text{HNO}_2 + \text{HNO}_2 \leftrightarrow \text{N}_2\text{O}_5 + \text{H}_2\text{O} \leftrightarrow \text{NO} + \text{NO}_2^+ + \text{H}_2\text{O}
\]

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

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

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 NO\textsuperscript{2−}, pH 6.5 and 1.5 mM NO\textsuperscript{2−}, 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 NO\textsuperscript{2−} decomposition (HNO\textsubscript{2}, NO\textsuperscript{2+}, and NO), only NO reacts with carboxy-PTIO. If

Figure 3
HNO\textsubscript{2} 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 NO\textsubscript{2−}. The CFU in the inoculum versus that after a 4-day anaerobic incubation were determined. The values for log\textsubscript{10}[CFU\textsubscript{inoculum}/CFU\textsubscript{after 4 days}] 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.

Table 1
Age-dependent emergence of mucA mutant P. aeruginosa from 5 different CF patients and sensitivity of each isolate to acidified NO\textsuperscript{2−}

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Mucoid</th>
<th>mucA mutation</th>
<th>Viability index</th>
<th>Note</th>
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<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>B</td>
<td>4.5</td>
<td>NM</td>
<td>No</td>
<td>0.13</td>
<td>First mucoid P. aeruginosa isolation</td>
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<tr>
<td></td>
<td>6.0</td>
<td>M</td>
<td>Yes, stop at 441</td>
<td>–3.1</td>
<td>First P. aeruginosa colonization</td>
</tr>
<tr>
<td>C</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>
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<tr>
<td></td>
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<td>M</td>
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<tr>
<td></td>
<td>5.5</td>
<td>M</td>
<td>Yes, stop at 351</td>
<td>–2.93</td>
<td>First P. aeruginosa colonization</td>
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<tr>
<td>D</td>
<td>0.5</td>
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<td>M</td>
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<tr>
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<td></td>
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<tr>
<td></td>
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<tr>
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<tr>
<td></td>
<td>5.7</td>
<td>M</td>
<td>No</td>
<td>0.9</td>
<td>First P. aeruginosa colonization</td>
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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.
HNO$_3$ reacted, the addition of carboxy-PTIO would cause no change in NO concentration. If NO$_2^-$ 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 HNO$_3$. 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 a direct or indirect contributor to the bactericidal activity of acidified NO$_2^-$. This conclusion is strongly supported by the observation that strain FRD1, but not FRD1/p$mucA$, 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 NO$_2^-$ 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 NO$_2^-$ 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 HNO$_3$ 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 HNO$_3$. For example, high NO levels promote formation of a strong nitrosating intermediate, N$_2$O$_3$ 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 O$_2$ could be problematic because of the attendant generation of O$_2^-$, which combines with NO to form peroxynitrite (ONOO$^-$), an extremely bactericidal species (32, 33). We used a sodAsodB mutant, devoid of iron- and manganese-cofacted superoxide dismutase. As such, it is extremely sensitive to even endogenously generated O$_2^-$. (34). Were trace oxygen to be reduced to O$_2^-$ 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 HNO$_3$ under anaerobic conditions. In contrast and as expected, under aerobic conditions, the sodAsodB mutant was more sensitive to HNO$_3$ (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 HNO$_3$ sensitivity of mucoid $mucA$ mutant bacteria. We first measured the activity of enzymes involved in P. aeruginosa anaerobic respiration, including NO$_2^-$ reductase (NAR), NIR, and NOR (Figure 5A). Strain FRD1 possessed approximately 3.7-fold greater NIR activity compared with nonmucoid FRD1/p$mucA$ and PAO1. However, strain FRD1 possessed only 4% and approximately 20% the NIR and NOR activity, respectively, of strain PAO1 (Figure 5A). Interestingly, FRD1/p$mucA$ 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 NO$_2^-$ to support anaerobic growth of this strain. In addition, the low NIR activity in strain FRD1 led to the constancy of HNO$_3$ levels in the culture medium and the attendant increase in NO levels compared with strains that metabolize NO$_2^-$. Strikingly, there was no significant loss of NO$_2^-$ from the culture medium after 4 days at pH 6.5, suggesting that there was no biological reduction of NO$_2^-$ in strain FRD1 and 16 different mucoid, $mucA$ mutant CF isolates assessed for NO$_2^-$ sensitivity (Supplemental Table 1) and remaining NO$_2^-$ levels (Figure 5B). Moreover, these data also demonstrate that NO formed from HNO$_3$ at this pH and NO$_2^-$ concentration in the presence of $mucA$ mutant organisms was virtually all recycled to NO$_2^-$. 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/p$mucA$ had little or no media NO$_2^-$ 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 HNO$_3$. Figure 5C demonstrates that a norCB mutant of strain FRD1 was approximately 10-fold more sensitive to acidified NO$_2^-$ than were wild-type organisms.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
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<th>$mucA$ mutation</th>
<th>Viability index</th>
<th>LD$_{50}$</th>
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<tr>
<td>PD0300 (PA01 $mucA22$)</td>
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<td>4.1 (± 0.2)</td>
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<td>PA01 $mucB$:Tc</td>
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<td>0.45</td>
<td>N/A</td>
</tr>
<tr>
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<td>0.39</td>
<td>N/A</td>
</tr>
<tr>
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<td>0.57</td>
<td>N/A</td>
</tr>
<tr>
<td>FRD1</td>
<td>NM</td>
<td>Yes</td>
<td>–3.84</td>
<td>2.8 (± 0.18)</td>
</tr>
<tr>
<td>FRD1 $algD$:Tn501</td>
<td>NM</td>
<td>Yes</td>
<td>&lt;–3.48</td>
<td>2.5 (± 0.1)</td>
</tr>
<tr>
<td>FRD1 $algT(U)$:Tn501</td>
<td>NM</td>
<td>Yes</td>
<td>&lt;–3.95</td>
<td>2.45 (± 0.15)</td>
</tr>
<tr>
<td>Clinical isolate 35</td>
<td>NM</td>
<td>Yes</td>
<td>&lt;–3.71</td>
<td>3.1 (± 0.08)</td>
</tr>
<tr>
<td>Clinical isolate 37</td>
<td>NM</td>
<td>Yes</td>
<td>&lt;–4.12</td>
<td>2.2 (± 0.1)</td>
</tr>
<tr>
<td>Clinical isolate 38</td>
<td>NM</td>
<td>Yes</td>
<td>&lt;–4.54</td>
<td>2.55 (± 0.34)</td>
</tr>
<tr>
<td>Clinical isolate 40</td>
<td>NM</td>
<td>Yes</td>
<td>&lt;–3.87</td>
<td>2.6 (± 0.2)</td>
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</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 LD$_{50}$ of NO$_2^-$ was determined from a graph correlating the percent survival of strains after 24 hours of exposure to acidified NO$_2^-$ ranging from 1–5 mM (see Figure 2E).
HNO₂ kills mucoid P. aeruginosa in in vitro anaerobic biofilms and fresh sputum isolates from CF patients. To explore the potential clinical application of HNO₂ in the treatment of mucoid P. aeruginosa infections in chronic CF patients, we first tested the effect of NO₂⁻ on the viability of biofilm bacteria. Anaerobic biofilms of strains FRD1 and FRD1/p mucA were grown for 1 day in media containing NO₃⁻, 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/p mucA (Figure 6, compare A and B). When the FRD1 biofilm was treated with NO₂⁻ 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 NO₂⁻ at pH 6.5 (Figure 6A, top right). In biofilms of FRD1/p mucA, however, resistance to acidified NO₂⁻ was clearly evident (Figure 6B).

We next monitored the effect of HNO₂ on the killing of sputum isolates from CF patients. Sputum isolates CF1, -2, -3, and -4 harbored nearly 100% mucoid mucA 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 NO₂⁻ sensitivity. When these isolates were exposed to 15 mM NO₂⁻ at pH 6.5 under anaerobic conditions for 1 day, a sharp decrease in viability was observed in the mucoid mucA 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 HNO₂ selectively kill mucoid mucA 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 HNO₂ 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 HNO₂ ex vivo. Figure 7A shows that mucoid bacteria were actually killed faster by HNO₂ 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 titers 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 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$_3^-$ was approximately 5 hours (Figure 8E), indicating that NO$_3^-$ 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 activities. 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$_3$ 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$_3^-$ 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 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 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.

**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{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, KNO\textsubscript{3} and/or NaNO\textsubscript{3} (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 NaNO\textsubscript{2} (for pH 5.5) to achieve the desired pH. Organisms surviving treatment with buffer and NO\textsubscript{2} were enumerated (31). NAR, NIR, and NOR activities were measured as described previously (21). Biofilm staining and image acquisition were accomplished as described previously (21).

\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).

**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 \(10^8\) FRD1 or FRD1/mucA entrapped in agar beads intratracheally as previously described (33). Following 24 hours’ incubation, mouse lungs were instilled with 25 μl of 15 mM NO\textsubscript{2} at pH 5.5 (in 0.1 M acetate buffer) or pH 6.5.
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
3
 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
3
. 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
3
 on the surface of cultured airway epithelia, NO
3
 levels were measured by the Griess reaction (54), and the percent rate of NO
3
 removal was calculated.

Preparation of sterile ultraparatenants 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

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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.