INTRODUCTION

As an obligate human pathogen, *Neisseria gonorrhoeae* is well adapted for growth on a variety of mucosal surfaces (Edwards & Apicella, 2004). During the course of infection, gonococci are expected to face an onslaught of oxidative and nitrosative stresses (Bogdan *et al*., 2000; Hampton *et al*., 1998). Previous research has shown that multiple gonococcal gene products aid in survival under stressful conditions (Potter *et al*., 2004, 2006; Stohl *et al*., 2005), and that hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$), nitric oxide (NO) and peroxynitrite (ONOO$^-$) are largely ineffective at eliminating infection (Alcorn *et al*., 1994; Barth *et al*., 2009; Edwards, 2010; Seib *et al*., 2003, 2004). Furthermore, studies have demonstrated that a subpopulation of gonococci survive within neutrophils, which are cells capable of generating reactive oxygen and nitrogen species (ROS and RNS, respectively) (Casey *et al*., 1986; Simons *et al*., 2005). This suggests that gonococci are capable of persistence within an activated immune system, and that oxygen-dependent mechanisms of bacterial killing may not be wholly effective at eradicating infection (Seib *et al*., 2006).

The concurrent presence of both O$_2^-$ and NO allows for the generation of peroxynitrite (PN), which is believed to be much more reactive than its parent molecules (Alvarez & Radi, 2003; Beckman & Koppenol, 1996; Goldstein & Merényi, 2008). PN reactivity is highly pH-dependent. At alkaline pH, the stable anion ONOO$^-$ is the predominant form of PN, while the more reactive acidic form of PN, ONOOH, increases in proportion with decreasing pH (reviewed by Beckman & Koppenol, 1996; Goldstein & Merényi, 2008). Physiological PN reactivity is rather complex and can be broadly categorized into two mechanisms: (1) direct oxidation of target molecules by PN, and (2) indirect effects of PN initiated by the radicals formed via decomposition of PN or PN-derived intermediates (Goldstein & Merényi, 2008). PN reactivity can lead to the oxidation of metal complexes, porphyrins, haem proteins, lipids and DNA (Burney *et al*., 1999; Goldstein & Merényi, 2008; Radi *et al*., 1991). PN can also cause the modification of amino acid residues within proteins, including the oxidation of thiol groups and nitration of tyrosine residues (Beckman & Koppenol, 1996; Pacher *et al*., 2007). In fact, PN is more or less capable of oxidizing any substrate, as the decomposition products of the acidic form of PN, NO$_2^-$, and especially 'OH, are highly oxidizing (Goldstein & Merényi, 2008).

We have reported that *Neisseria gonorrhoeae* is extremely resistant to reactive nitrogen species (RNS) including peroxynitrite (PN). Recent literature suggests that catalase can provide protection against commercial preparations of PN. Though wild-type gonococci were shown to be highly resistant to 2 mM PN, *Neisseria meningitidis* and a gonococcal katA mutant were both shown to be extremely sensitive to 2 mM PN. Analysis of translational fusions to lacZ of the catalase promoters from *N. gonorrhoeae* and *N. meningitidis* demonstrated that basal katA expression from gonococci is 80-fold higher than in meningococci, though meningococcal katA retains a greater capacity to be activated by OxyR. This activation capacity was shown to be due to a single base pair difference in the −10 transcription element between the two kat promoters. PN resistance was initially shown to be associated with increasing catalase expression; however, commercial preparations of PN were later revealed to contain higher levels of contaminating hydrogen peroxide (H$_2$O$_2$) than expected. Removal of H$_2$O$_2$ from PN preparations with manganese dioxide markedly reduced PN toxicity in a gonococcal katA mutant. Simultaneous treatment with non-lethal concentrations of PN and H$_2$O$_2$ was highly lethal, indicating that these agents act synergistically. When treatment was separated by 5 min, high levels of bacterial killing occurred only when PN was added first. Our results suggest that killing of *N. gonorrhoeae ΔkatA* by commercial PN preparations is likely due to H$_2$O$_2$, that H$_2$O$_2$ is more toxic in the presence of PN, and that PN, on its own, may not be as toxic as previously believed.
PN-mediated killing has been shown in a number of bacterial species (Alam et al., 2006; Dyet & Moir, 2006; Kuwashara et al., 2000; Yu et al., 1999; Zhu et al., 1992). Analysis of PN-mediated killing in bacteria can be complicated due to the extremely short half-life of PN in neutral aqueous solution (~1 s), as well as variation in media components that may react with PN to form different reactive species (Goldstein & Merenyi, 2008; Schmidt et al., 1998). The use of molecular generators to generate PN through the production of O$_2^-$ and NO complicates interpretations of data due to the simultaneous presence of multiple reactive species. A few earlier studies have suggested that catalase may be able to act as a peroxynitritase, and thus act catalytically to detoxify PN (Gebicka & Didik, 2009; McLean et al., 2010; Sahoo et al., 2009; Wengenack et al., 1999). We have previously reported that *N. gonorrhoeae* demonstrates marked resistance to 2 mM concentrations of commercially prepared PN; however, we were unable to determine the source of this resistance (Barth et al., 2009). In this study, we initially determine that gonococcal catalase, KatA, provides resistance to commercially prepared preparations of PN. However, we subsequently determine that commercial PN preparations are contaminated with H$_2$O$_2$ (a substrate used in the organic synthesis of PN), and that H$_2$O$_2$ likely contributed to what has been reported as ‘PN-mediated’ killing. We provide evidence that bicarbonate provides some protection against both PN- and H$_2$O$_2$-mediated killing, and that PN can sensitize bacteria to H$_2$O$_2$ toxicity, even in the presence of bicarbonate.

**METHODS**

**Bacterial strains and growth conditions.** All gonococcal mutant strains were derived from laboratory strain F62 (Table 1). *N. gonorrhoeae* and *Neisseria meningitidis* strain MC58 were grown on Difco GC medium base (Becton Dickinson) plates with 1 % Kellogg’s supplement (GCK) (Kellogg et al., 1963), in a 5 % CO$_2$ incubator at 37 °C. Broth cultures were grown in GCP broth [proteose peptone no. 3 (Difco, 15 g), soluble starch (1 g), KH$_2$PO$_4$ (4 g), K$_2$HPO$_4$ (1 g), NaCl (5 g) per litre of distilled H$_2$O] supplemented with 1 % Kellogg’s, and 0.042 % sodium bicarbonate (GCK broth), with shaking at 250 r.p.m. *Escherichia coli* DH10B was grown on plates using either GCK agar or LB agar (Bacto tryptone (Difco, 10 g), yeast extract (Difco, 5 g), NaCl (10 g), Bacto agar (Difco, 15 g) per litre). *E. coli* broth cultures were grown in GCK or LB broth.

**Chemicals and reagents.** PN (Calbiochem), isoamyl nitrite (Acros), and H$_2$O$_2$ (Acros) were used in these studies. PN was also synthesized as described elsewhere (Uppu, 2006), with the following exception; H$_2$O$_2$ removal was accomplished by stirring PN solutions in the presence of granular MnO$_2$ followed by filtration, rather than running PN solutions through a MnO$_2$-packed column.

**Survival counts following treatment with H$_2$O$_2$ and PN.** Broth cultures of *N. gonorrhoeae* were grown to an OD$_{560}$ of 0.4–0.6 and diluted back to OD$_{560}$ 0.2 in GCK broth. For simultaneous PN/H$_2$O$_2$ treatment, 1 ml of this culture was added to a culture tube containing PN (final concentration 2.0 mM) and H$_2$O$_2$ (final concentration 2.0 mM) and H$_2$O$_2$ (final concentration 2.0 mM) and H$_2$O$_2$ (final concentration 2.0 mM) and H$_2$O$_2$ (final concentration 2.0 mM) and H$_2$O$_2$ (final concentration 2.0 mM) and H$_2$O$_2$ (final concentration 2.0 mM).

**Table 1.** Bacterial strains and constructs used in this study

<table>
<thead>
<tr>
<th>Plasmid, strain or construct</th>
<th>Relevant genotype or properties</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td>Plasmids</td>
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<tr>
<td>pVII151</td>
<td>pLES94/F62 katA upstream</td>
<td>This study</td>
</tr>
<tr>
<td>pVII152</td>
<td>pLES94/MC58 katA upstream</td>
<td>This study</td>
</tr>
<tr>
<td>pVII153</td>
<td>pLES94/F62 katA upstream (TATAAT→TATAGT)</td>
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<td></td>
<td></td>
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<tr>
<td>E. coli strain</td>
<td>F$^{-}$ endA1 recA1</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neisseria strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC58*</td>
<td><em>N. meningitidis</em> (serogroup B)</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>F62</td>
<td>pro$^{-}$ (<em>N. gonorrhoeae</em> parental)</td>
<td>Laboratory collection</td>
</tr>
<tr>
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</tr>
<tr>
<td>RUG7961</td>
<td>F62 transformed with pVII152</td>
<td>This study</td>
</tr>
<tr>
<td>RUG7962</td>
<td>F62 transformed with pVII1453</td>
<td>This study</td>
</tr>
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<td>RUG7963</td>
<td>F62, insertion inactivation of katA</td>
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</tr>
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<td>RUG7964</td>
<td>F62 transformed with pVII154</td>
<td>This study</td>
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</tr>
<tr>
<td>RUG7966</td>
<td>F62 transformed with pVII156</td>
<td>This study</td>
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</tbody>
</table>

*All other strains indicated in this section are derived from *N. gonorrhoeae*.

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0.2 mM). For sequential PN/H₂O₂ treatment, either PN (2 mM) or 
H₂O₂ (0.2 mM) was added to culture tubes before the addition of 1 ml gonococcal culture. The other reactive species was added 5 min 
later. The tubes were incubated at 37 °C for 45 min, and the bacteria 
were diluted and plated to determine viability. Survival was measured by 
dividing the final c.f.u. by the initial c.f.u. To treat cultures with reactive species in the absence of bicarbonate, cultures were filtered 
through 0.2 µm pore-size, 47 mm diameter Nucleopore polycarbonate 
Track-Etch membranes (Whatman) and rinsed with two volumes of fresh medium (without bicarbonate). The filter was then 
added to a tube containing fresh medium and the cells were 
resuspended. This culture was then diluted back to OD₆₀₀ 0.2 and 
treated as described above.

Gonococcal transformation. A light suspension of type 1 cells 
(Kellogg et al., 1963) was prepared in 1 ml GCK broth containing 
0.042 % NaHCO₃ and 10 mM MgCl₂. Purified plasmid DNA or 
ligation mixture was added, and 100 µl of the suspension was plated 
onto two GCK plates that were incubated right side up for between 6 
and 9 h at 37 °C. Cells were then harvested from the plates and 
streaked on GCK plates containing the appropriate antibiotic for 
selection of clones. The ktaA mutation in strain F62 was created by 
transformation of F62 with genomic DNA from a ktaA mutant strain 
derived from gonococcal strain FA1090 (Soler-García & Jerse, 2004).

PCR and cloning. Genomic DNA from gonococcal strain F62, 
meningococcal strain MC58 and E. coli strain DH10B was isolated for 
use as a PCR template. Promoter sequences for lacZ fusions, kta gene 
cassettes and chromosomal regions for insertional inactivation of 
genes were amplified with iProof High-Fidelity DNA polymerase 
(Bio-Rad). Clones were screened by PCR for the presence and 
orientation of the insert using AmpliTaq (Applied Biosystems). Primer sequences used for all constructs are available from the 
authors upon request.

Construction of lacZ fusions. Translational lacZ fusions were 
constructed with pLES94 (Silver & Clark, 1995), using genomic DNA 
from F62 or MC58 as the template. PCR fragments and pLES94 were 
cut with BamHI. The digested insert and plasmid were ligated and 
cloned into E. coli DH10B. Transformants were selected on LB 
medium plates containing chloramphenicol (25 µg ml⁻¹) and X-Gal 
(40 µg ml⁻¹; Invitrogen). For site-specific mutagenesis of the 
gonococcal ktaA –10 element, splice overlap extension PCR was 
used. A compensatory base pair change in nucleotide 158 from 
Calbiochem, N. gonorrhoeae strain F62 exhibited a 
high level of resistance compared with N. meningitidis 
strain MC58 (~1.0 vs ~6.5 logs of killing, respectively; Fig. 
1). Recent reports have suggested a role for catalase in the 
catalytic breakdown of PN, and high catalase activity is a 
hallmark feature of N. gonorrhoeae strains (Bisaillon et al., 
1985; Soler-García & Jerse, 2004). Mid-exponential 
cultures of N. gonorrhoeae were shown to contain 25-fold 
higher catalase activity than those of N. meningitidis (Fig. 
1). We therefore reasoned that the high basal catalase 
activity in gonococci may be responsible for the difference in 
PN resistance between these closely related species. 

A gonococcal ktaA mutant was constructed and tested for 
PN resistance. The ktaA mutant was almost completely 
killed by 2 mM PN (Fig. 1). PN resistance could be 
restored by IPTG induction of a cloned ktaA gene under the 
control of the P₂₄₄ promoter in a ΔktaA mutant. These 
data demonstrate that KatA protects N. gonorrhoeae against 
killing by commercial PN preparations, and that the 
susceptibility of N. meningitidis to such preparations may 
be due to its lower basal catalase activity.

Differences in the neisserial ktaA upstream 
region are responsible for the difference in basal 
catalase activity and OxyR induction capacity 

Despite the high sequence similarity between the gonococcal 
and meningococcal OxyR proteins (only two amino acid
substitutions), as well as a high level of sequence conservation in the katA upstream region (Fig. 2), previous reports have demonstrated that a large difference in OxyR-mediated regulation of the katA gene in these two species exists (Ieva et al., 2008; Tseng et al., 2003). In N. meningitidis, katA expression has been shown to be repressed by reduced OxyR,
and to be activated by H$_2$O$_2$-induced OxyR oxidation, with OxyR remaining DNA-bound regardless of its oxidation state (Ieva et al., 2008). In N. gonorrhoeae, it has been suggested that OxyR acts primarily as a repressor of katA expression, as the catalase activity of a ΔoxyR mutant is greatly increased compared with that of wild-type gonococci (Tseng et al., 2003).

To determine the basis for the difference in basal catalase activity and katA regulation between these two pathogenic species, translational promoter–lacZ fusions to the protein itself.

Data represent the mean ± SD in Miller units of 12 determinations.

<table>
<thead>
<tr>
<th>Promoter fusion to lacZ*</th>
<th>Parental</th>
<th>ΔoxyR</th>
<th>Parental + H$_2$O$_2$</th>
<th>Fold repression by OxyR†</th>
<th>Fold activation by OxyR‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>P$_\text{katF62}$</td>
<td>430 ± 70</td>
<td>7000 ± 570</td>
<td>960 ± 64</td>
<td>16.1</td>
<td>2.2</td>
</tr>
<tr>
<td>P$_\text{katMC58}$</td>
<td>9 ± 2</td>
<td>140 ± 18</td>
<td>410 ± 62</td>
<td>15.4</td>
<td>43.1</td>
</tr>
<tr>
<td>P$_\text{katF62 TATAAT→TATAGT}$</td>
<td>15 ± 2</td>
<td>220 ± 27</td>
<td>550 ± 94</td>
<td>14.4</td>
<td>36.7</td>
</tr>
</tbody>
</table>

*All promoter fusions to the lacZ gene were assayed in gonococcal strain F62.
†Fold OxyR-mediated repression measured by the expression level in a ΔoxyR strain divided by the basal expression from the parental promoter.
‡Fold OxyR-mediated activation measured by the promoter expression following H$_2$O$_2$ treatment divided by the basal expression from the parental promoter.

**Table 2. β-Galactosidase activity of kat::lacZ fusions**

Induction of catalase activity confers protection against commercial PN preparations in a dose-dependent manner

At this point, it appeared that katA had been responsible for the detoxification of the Calbiochem PN preparation, and thus the observed resistance to PN-mediated killing. To determine whether this was a novel function of katA or a general role for catalase proteins, gonococcal katA, as well as E. coli catalase KatE, and catalase-peroxidase KatG, were placed under the control of an IPTG-inducible P$_\text{lac}$ promoter, and expression was induced in gonococcal strain F62 ΔkatA to test the ability of these catalases to protect against PN-mediated killing (Fig. 3). E. coli KatE and KatG both had catalase activity when expressed in gonococci, though the activity was not as high as the level observed from induction of gonococcal KatA (Fig. 3a). This was likely due to differences in catalase transcription and/or translation, and not to gross differences in specific activity between the enzymes, as the quantity of these catalase
proteins at maximal induction levels (1 mM IPTG), as determined by Coomassie staining, appeared to correlate with the observed catalase activity of the culture (data not shown).

Catalase activity in gonococcal strain F62 ΔkatA harbouring a katA, katE or katG gene under the control of P_{lac} was induced to varying degrees using different concentrations of IPTG, and survival was determined following exposure to 2 mM Calbiochem PN (Fig. 3b). Increasing catalase activity was shown to correlate with an increase in survival against PN challenge in a dose-dependent manner. These data show that catalase activity protects cells from killing by the PN solution, regardless of the particular catalase protein from which the activity is derived.

Calbiochem PN solution is contaminated with H_{2}O_{2}

At this point, these data were consistent with catalase playing a role in protection against PN-mediated killing. Unfortunately, there was an approximately 6 month hiatus in Calbiochem’s production of the PN solutions that we required to complete our studies. However, the method used by Calbiochem to synthesize PN was relatively simple, and could be completed in less than an hour. Briefly, PN synthesis was performed by the addition of isoamyl nitrite and H_{2}O_{2} in a basic homogeneous solvent system, followed by the removal of unreacted H_{2}O_{2} by treatment with granular manganese dioxide (MnO_{2}) (Uppu, 2006). PN production was easy to observe, as PN solutions have a characteristic yellow colour, and PN concentration is easy to quantify at its peak absorbance of 302 nm (ε=1670 M^{-1} cm^{-1}) (Hughes & Nicklin, 1970).

Unlike the Calbiochem PN solution, PN solutions that were prepared in our laboratory failed to cause significant killing of the gonococcal ΔkatA strain at concentrations up to 2 mM (Fig. 4a). However, when the dose–response curve was repeated in the presence of a sublethal concentration of H_{2}O_{2}, killing increased with PN concentration, reaching levels observed with the commercial preparation. When dose–response curves for H_{2}O_{2} were performed in the presence and absence of PN (Fig. 4b), PN caused a sharp threshold drop in survival. N. meningitidis was also found to survive challenge to PN solutions that we prepared (data not shown). These data suggest that what had previously been reported to be PN-mediated killing (Barth et al., 2009) of the sensitive N. meningitidis may have actually been due to the combinatorial effect of both PN and H_{2}O_{2}, and that these compounds act synergistically to cause cell killing.

Effects of bicarbonate on PN/H_{2}O_{2}-mediated killing

Gonococci require carbon dioxide for growth, which is supplied in broth culture in the form of sodium bicarbonate.
However, carbon dioxide is known to react with both PN and H$_2$O$_2$ (Augusto et al., 2002; Richardson et al., 2000). To determine the effect of carbon dioxide on PN/H$_2$O$_2$-mediated cell killing, the gonococcal ΔkatA strain was subjected to PN or H$_2$O$_2$ treatment in the presence (Fig. 5a) or absence of bicarbonate (Fig. 5b). In the absence of bicarbonate, cells displayed increased sensitivity to the individual addition of PN or H$_2$O$_2$ (~1 log and ~2 logs of killing, respectively). There was relatively little increase in sensitivity to H$_2$O$_2$ and PN when these agents were added in combination (~5 logs of killing, compared with ~6.5 with bicarbonate, within experimental error). This suggests that while bicarbonate decreases sensitivity to these reactive species individually, it has no effect on combinatorial PN/H$_2$O$_2$ killing. In turn, this would also suggest that it is unlikely that the bicarbonate radical plays a role in synergistic PN/H$_2$O$_2$ killing.

**Effect of timing on PN/H$_2$O$_2$-mediated killing**

We next wished to determine whether there was an effect on PN/H$_2$O$_2$-mediated killing when these two agents were added sequentially rather than simultaneously. When PN was added 5 min before H$_2$O$_2$ addition, we observed approximately 3 logs of cell killing (Fig. 6). When PN was added 5 min after H$_2$O$_2$ addition, we observed only an approximate single log of killing. These data show that the order of addition of these agents can influence the PN/H$_2$O$_2$-mediated killing effect, which suggests that PN can influence the sensitivity of the organism to H$_2$O$_2$.

**DISCUSSION**

We have previously reported that *N. gonorrhoeae* is highly resistant to the toxic effects of PN (Barth et al., 2009). This resistance is seen whether the PN is generated by an NO donor plus a superoxide generator or by direct addition of PN. The gonococcus is not sensitive to NO and grows aerobically in its presence using the NO as a terminal electron acceptor. We were unable to identify a basis for the high level of PN resistance, as mutations in the gonococcal genes orthologous to known RNS resistance genes had no effect on the inability of PN to kill *N. gonorrhoeae*.

The effects of PN have been studied in many different organisms under various conditions. The mechanism of toxicity of PN seems to differ between organisms, showing the importance of studying PN in individual microbial species/strains. In *E. coli*, PN has been shown to be more toxic than NO. H$_2$O$_2$, either by pre-treatment, or with simultaneous addition, enhances this toxic effect (Brunelli et al., 1995). Previous experiments with *E. coli* and PN showed that metal ion chelators did not affect toxicity, nor did the addition of hydroxyl radical scavengers. Addition of DMSO did enhance killing by PN, for the hypothesized reason of increased NO$_2$ production (Zhu et al., 1992). A
A study examining differences in sensitivity to RNS and ROS between *E. coli* and *N. meningitidis* showed that in a xanthine/xanthine oxidase paired system, addition of NO increased toxicity to *E. coli*, while decreasing toxicity in *N. meningitidis* (Dyet & Moir, 2006). Studies involving mycobacteria have had similar results. A study of various strains of mycobacteria, including *Mycobacterium tuberculosis*, showed that while all species were susceptible to NO and NO₂, virulent strains showed greater resistance to PN than avirulent strains (Yu et al., 1999). These studies emphasize the ways in which ROS and RNS can have diverse effects on different species. One important reaction for mediating the toxicity of PN is its rapid reaction with CO₂. The effects of CO₂ on PN toxicity in *Trypanosoma cruzi* demonstrate that the rapid reaction of CO₂ with PN decreases its effective toxicity based on the distance that the PN needs to diffuse (Alvarez et al., 2004). The CO₂ reduces the ability of PN to reach its target, and therefore to exert a toxic effect. Another study showed the protective effect of CO₂ on PN toxicity, by comparing *Helicobacter pylori* cultures exposed to PN with and without urea (Kuwahara et al., 2000). Urease breaks down urea into CO₂ and NH₃, and when urea was added to a culture that produced urease, the bacteria no longer showed sensitivity to PN. Urea added to bacteria which did not produce urease did not demonstrate this protective effect.

Earlier reports have provided evidence that in some organisms, catalase contains PN reductase ability (McLean et al., 2010; Wengenack et al., 1999). Previously, we demonstrated resistance to PN in *N. gonorrhoeae*, while its close relative, *N. meningitidis*, was extremely sensitive (Barth et al., 2009). As the difference in catalase expression is one of the major distinctions between these two organisms (Seib et al., 2004), we reasoned that catalase could be involved in PN resistance. We were able to show that the gonococcal catalase, KatA, can protect cells against PN, as increasing catalase activity led to an increase in cell survival against PN challenge in a dose-dependent manner. At first, these data suggested that KatA had a directly protective role against PN toxicity. When there was an interruption of our ability to obtain commercially prepared PN, we began to synthesize it in our laboratory. Through treatment with MnO₂, we removed excess H₂O₂, a process that should have been performed during commercial preparation as well (Uppu & Pryor, 1996). After performing this treatment, we no longer saw substantial amounts of cell killing, though toxicity could be restored by the addition of sublethal levels of H₂O₂. This suggests that the
large degree of killing observed previously was most likely due to \( H_2O_2 \) contamination, and that the protective effect of \( katA \) expression observed in this study was due to the reduction of \( H_2O_2 \).

Even small amounts of \( H_2O_2 \) demonstrated synergistic killing of a \( katA \) mutant in combination with PN. Killing was observed with \( H_2O_2 \) at 0.2 mM and PN at 2.0 mM, concentrations that did not result in substantial killing individually. Researchers must be careful when using commercial preparations of PN, as these data suggest that even small concentrations of \( H_2O_2 \) may result in drastic changes in cell survival when combined with PN. It is important to note that the product insert included with Calbiochem PN lists contaminating levels of both nitrite and nitrate, yet makes no mention of residual \( H_2O_2 \) contamination.

\( H_2O_2 \) can freely diffuse across membranes, but does not have a directly toxic effect; instead, it participates in a variety of intracellular reactions (Bienert et al., 2006). In wild-type strains of \( N. gonorrhoeae \), the major reaction will be with catalase (\( 2H_2O_2 \rightarrow 2H_2O + O_2 \)). \( H_2O_2 \) also reacts with thiol groups within proteins, forming disulfide bridges (Zheng et al., 1998). This reaction can be reversed through the activity of disulfide reductases (Prinz et al., 1997). The major source of \( H_2O_2 \) toxicity is related to its ability to react with iron to form hydroxyl radicals (\('OH\)), which are much more dangerous and can lead to potentially lethal DNA mutation events (Inoue & Kawanishi, 1987).

Earlier studies have shown a relationship between killing by ROS and exposure to RNS. One experiment studied the effect of acidified nitrite ion addition on \( H_2O_2 \) killing in various micro-organisms, including bacteria, fungi and amoebae (Heaselgrave et al., 2010). They found that acidified nitrite substantially increased the killing effect of \( H_2O_2 \) (~4 logs). This was hypothesized to be due to the formation of \( PN \) ions in the solution, increasing the toxicity of \( H_2O_2 \) through an unknown mechanism. A second study demonstrated increased \( H_2O_2 \) killing of \( E. coli \) by the addition of nitrite ion in a lactate buffer (Kono et al., 1994). This was hypothesized to be due to the formation of PN from nitrite in the presence of \( H_2O_2 \), which then breaks down into NO\(_2\), which was concluded to be the toxic molecule. A separate study showed that NO could also enhance killing by \( H_2O_2 \) in \( E. coli \) (Pacelli et al., 1995). Those authors hypothesized many pathways that could lead to increased toxicity due to NO addition. One pathway was the formation of NO\(_x\) species, which would in turn react with thiol groups on proteins and glutathione, decreasing the reduction potential of the cell, and increasing its sensitivity to powerful oxidizers such as \( H_2O_2 \). A second hypothesized pathway was through PN formation, causing a release of metal ions from metalloproteins. These metal ions subsequently react to form potent ROS that cause lethal DNA damage.

We hypothesize that in the presence of CO\(_2\) and PN readily decomposes into CO\(_3^−\) and NO\(_2\) (Augusto et al., 2002). NO\(_2\) is able to pass through the cell membrane and convert thiol groups of proteins or glutathione into nitrosothiols (Zhang & Hogg, 2004). Once converted, these groups are much less reactive and, more importantly, they no longer react with and consume \( H_2O_2 \). It has been reported that PN preferentially targets iron–sulfur clusters within bacteria, causing a release of intracellular iron (Pacelli et al., 1995). Because \( ΔkatA \) strains and \( N. meningitidis \) have much reduced ROS-scavenging ability compared with wild-type gonococci, \( H_2O_2 \) would be available to react with this free iron, causing an increase in production of deadly \( OH \) (Inoue & Kawanishi, 1987). Gonococci have high intracellular levels of glutathione and glutathione reductase (Archibald & Duong, 1986; Seib et al., 2006). Glutathione would normally protect gonococci against oxidative stress through oxidation-reduction cycling, but when S-nitrosylated by NO\(_2\), it would no longer be capable of providing this protection. This may explain the effect of changing the order of addition of these agents. When PN and \( H_2O_2 \) are added simultaneously, PN, which was added in a 10-fold molar excess over \( H_2O_2 \), converts thiol groups to S-NO\(_2\) groups, and also causes a release of iron from Fe–S clusters-containing proteins. When this occurs, \( H_2O_2 \) is unable to oxidize glutathione. Instead, \( H_2O_2 \) is available to react with free iron, leading to \( OH \) production. In the case when PN...
was added before the $H_2O_2$, the PN could have nitrosylated thiol groups and released iron from iron–sulfur clusters. The iron may have been scavenged by the cell during the 5 min delay, causing a slightly reduced, though still pronounced, killing effect (Pacelli et al., 1995). When $H_2O_2$ was added before the PN, however, it could be rapidly scavenged by reduced thiols. This residual scavenging activity reduces the concentration of $H_2O_2$ before PN addition. When PN is added at this time, a decreased $H_2O_2$ concentration leads to the decrease in killing. Alternatively, $H_2O_2$ oxidation of thiols may make them no longer targets of PN nitrosylation and thus they may be more easily reduced by various reductases. That would leave only release of iron from iron–sulfur as the mechanism of increase in $H_2O_2$ sensitivity by PN. PN, on its own, appears to have a growth inhibitory rather than cytotoxic effect. This study provides evidence that previous reports of PN-mediated toxicity may have been due to $H_2O_2$ contamination, and that researchers should keep this in mind when purchasing such products, even from large and reputable chemical companies. Taken together, our data suggest that PN, by itself, may not be as toxic as previously thought.

At this time we are still unable to explain the mechanism of resistance to PN-mediated killing in gonococci when treated solely with this RNS. Although we are unable to rule out the possibility that KatA has PN reductase activity, our data suggest that this protein is not required for PN resistance. However, gonococci may also encode an as-yet-unidentified PN reductase. As gonococci encode a truncated denitrification pathway (Barth et al., 2009), it is possible that basal expression of denitrification genes may aid in removing toxic RNS or perhaps even prevent their formation. It is also possible that gonococci encode novel repair/detoxification pathways that are able to provide protection against RNS and which are not encoded by more sensitive organisms like E. coli (Brunelli et al., 1995). It is also possible that gonococci have repair mechanisms analogous to those of E. coli, but with greater efficiency or higher cellular concentration. Indeed, a high intracellular glutathione concentration is a hallmark feature of this organism (Archibald & Duong, 1986). As an obligate human pathogen that has become extremely well adapted to its host, it may also be the case that the gonococcus simply does not have any target(s) of PN-mediated killing or has modified target(s), so that it is no longer affected by PN.

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