The role of catalase in gonococcal resistance to peroxynitrite

Stephen A. Spence, Virginia L. Clark and Vincent M. Isabella

Correspondence

Virginia L. Clark

Ginny_Clark@urmc.rochester.edu

Department of Microbiology and Immunology, School of Medicine and Dentistry,
University of Rochester, Box 672, 601 Elmwood Ave, Rochester, NY 14642, USA

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_2O_2) than expected. Removal of H_2O_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_2O_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* $\Delta katA$ by commercial PN preparations is likely due to H_2O_2 , that H_2O_2 is more toxic in the presence of PN, and that PN, on its own, may not be as toxic as previously believed.

Received 31 July 2011

Revised 12 October 2011

Accepted 17 November 2011

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.*, 2009a, b; Seib *et al.*, 2004, 2006; Stohl *et al.*, 2005), and that hydrogen peroxide (H_2O_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 $\cdot\text{OH}$, are highly oxidizing (Goldstein & Merényi, 2008).

Abbreviations: PN, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species.

PN-mediated killing has been shown in a number of bacterial species (Alam *et al.*, 2006; Dyet & Moir, 2006; Kuwahara *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 & Merényi, 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_2O_2 (a substrate used in the organic synthesis of PN), and that H_2O_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_2O_2 -mediated

killing, and that PN can sensitize bacteria to H_2O_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_2PO_4 (4 g), K_2HPO_4 (1 g), NaCl (5 g) per litre of distilled H_2O] 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_2O_2 (Acros) were used in these studies. PN was also synthesized as described elsewhere (Uppu, 2006), with the following exception; H_2O_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_2O_2 and PN. Broth cultures of *N. gonorrhoeae* were grown to an OD_{600} of 0.4–0.6 and diluted back to OD_{600} 0.2 in GCK broth. For simultaneous PN/ H_2O_2 treatment, 1 ml of this culture was added to a culture tube containing PN (final concentration 2.0 mM) and H_2O_2 (final concentration

Table 1. Bacterial strains and constructs used in this study

Ap^r, Cm^r, Erm^r and Kn^r, ampicillin, chloramphenicol, erythromycin and kanamycin resistance, respectively.

Plasmid, strain or construct	Relevant genotype or properties	Reference or source
Plasmids		
pLES94	Promoterless <i>lacZ</i> vector; Ap ^r , Cm ^r	Silver & Clark (1995)
pVI151	pLES94/F62 <i>kata</i> upstream	This study
pVI152	pLES94/MC58 <i>kata</i> upstream	This study
pVI153	pLES94/F62 <i>kata</i> upstream (TATAAT→TATAGT)	This study
pGCC4	Gonococcal complementation vector, <i>lacI</i> , <i>P_{lac}</i> , Kn ^r , Erm ^r	Mehr & Seifert (1998)
pVI154	pGCC4/ <i>kata</i> (<i>N. gonorrhoeae</i>)	This study
pVI155	pGCC4/ <i>katE</i> (<i>E. coli</i>)	This study
pVI156	pGCC4/ <i>katG</i> (<i>E. coli</i>)	This study
<i>E. coli</i> strain		
DH10B	F [−] <i>endA1 recA1</i>	Laboratory collection
<i>Neisseria</i> strains		
MC58*	<i>N. meningitidis</i> (serogroup B)	Laboratory collection
F62	<i>pro</i> [−] (<i>N. gonorrhoeae</i> parental)	Laboratory collection
RUG7960	F62 transformed with pVI151	This study
RUG7961	F62 transformed with pVI152	This study
RUG7962	F62 transformed with pVI1453	This study
RUG7963	F62, insertional inactivation of <i>kata</i>	This study
RUG7964	F62 transformed with pVI154	This study
RUG7965	F62 transformed with pVI155	This study
RUG7966	F62 transformed with pVI156	This study

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

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 I 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 *kata* mutation in strain F62 was created by transformation of F62 with genomic DNA from a *kata* 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, *kat* 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 *Bam*HI. 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 *kata* -10 element, splice overlap extension PCR was used. A compensatory base pair change in nucleotide -8 was incorporated into internal primers to change the -8 nucleotide from A to G. Plasmids were checked for the presence and orientation of the insert by PCR, and those plasmids that contained an insert in the correct orientation were used to transform F62. Colony PCR was performed on chloramphenicol-resistant colonies to confirm the presence of the reporter construct. PCR products were also sequenced to ensure that the appropriate fusion had been made.

β-Galactosidase assays. Gene reporter activity was determined by β-galactosidase assays from wild-type and Δ*oxyR* strains grown in the presence or absence of 2 mM H₂O₂. Gonococcal cells were grown in GCK broth to OD₆₀₀ 0.5–0.7 and incubated with H₂O₂ (2 mM) for 1 h when appropriate. Cells were pelleted and resuspended in Z buffer (Miller, 1972), lysed with chloroform and 0.1 % SDS, and assayed as described by Miller (1972). Activity was reported in Miller units and the results were reported as the mean of at least six assays performed in duplicate from each day that the cultures were grown.

Construction of inducible gonococcal and *E. coli* *kat* strains. Gonococcal *katA*, and *E. coli* *katE* and *katG* gene cassettes, which included the wild-type ribosome-binding sites, were PCR-amplified and treated with T4 polynucleotide kinase (NEB) to add phosphates to the ends of the PCR products. These fragments were ligated to

PmeI-cut gonococcal complementation vector pGCC4 (Mehr & Seifert, 1998), and the ligation mix was used to transform chemically competent *E. coli* strain DH10B. Clones were selected on LB plates containing kanamycin (Calbiochem) at 50 µg ml⁻¹. Restriction mapping was used to determine the presence and proper orientation of inserts. The plasmid was isolated from *E. coli* clones and used to transform gonococcal strain F62. Gonococcal clones harbouring chromosomal integrations of the pGCC4-based constructs were selected on erythromycin at 2 µg ml⁻¹.

Catalase assays. Catalase activity in whole cells of *N. gonorrhoeae* and *N. meningitidis* was measured by reduction in H₂O₂ concentration, using an Apollo 4000 free radical analyser with an ISO-HPO-100 hydrogen peroxide sensor (World Precision Instruments). A standard curve for conversion of pA to nM H₂O₂ was generated as per the manufacturer's instructions. All standard curves and enzyme reactions were performed in temperature-controlled reaction vessels (World Precision Instruments) at a constant temperature of 30 °C. Cells were grown aerobically and suspended in GCK broth for 10 min, after which H₂O₂ was added to a final concentration of 100 µM. The H₂O₂ concentration was measured continuously, and data were exported into Microsoft Excel to determine reaction rates. The absorbance of the bacterial suspension was determined in a Beckman DU 640 spectrophotometer. Specific activity is expressed as units (nmoles H₂O₂ decomposed min⁻¹) per OD₆₀₀ unit.

RESULTS

Catalase provides protection against PN preparations

When exposed to a 2 mM concentration of PN obtained 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 *katA* mutant was constructed and tested for PN resistance. The *katA* mutant was almost completely killed by 2 mM PN (Fig. 1). PN resistance could be restored by IPTG induction of a cloned *katA* gene under the control of the *P_{lac}* promoter in a Δ*katA* 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 *katA* 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

Fig. 1. Survival following 2 mM PN exposure and catalase activity of *N. gonorrhoeae* and *N. meningitidis* strains. All cultures were grown to exponential phase and diluted to OD_{600} 0.2. One millilitre was exposed to 2 mM PN and grown out for 45 min before plating to determine viability (a), and 2 ml was used to determine catalase activity (b). For strains incubated with IPTG, cultures were exposed to 0.2 mM IPTG for 90 min before determination of survival and catalase activity. Strains: F62 (parental), $\Delta katA$ ($\Delta katA$), $katA-/+$ ($\Delta katA$ strain containing a chromosomal insertion of a P_{lac} -inducible *katA* allele), MC58 (meningococcal strain). Results are representative of at least three independent determinations; error bars, SD.

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,

Fig. 2. Schematic representation of the gonococcal and meningococcal *katA* upstream regions. The OxyR binding site, promoter elements and transcription start site are based on those described for meningococcal *katA* (Ieva *et al.*, 2008).

and to be activated by H₂O₂-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 Δ *oxyR* 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 gonococcal F62 and meningococcal MC58 *katA* promoters were constructed and analysed in an F62 background (Table 2). The strain expressing P_{kat}F62::*lacZ* displayed higher β -galactosidase activity than the strain expressing P_{kat}MC58::*lacZ*, consistent with a higher basal catalase activity in gonococci (Fig. 1, Table 2). Though β -galactosidase activity was extremely high in a Δ *oxyR* strain expressing P_{kat}F62::*lacZ* compared with a Δ *oxyR* strain expressing P_{kat}MC58::*lacZ*, both promoters appeared to be equally repressed by OxyR (16.1-fold vs 15.4-fold, respectively; Table 2). The largest difference in expression patterns between the two promoters was observed upon treatment with 2 mM H₂O₂. Whereas the strain expressing P_{kat}MC58::*lacZ* was induced 43.1-fold over the basal promoter activity, the strain expressing P_{kat}F62::*lacZ* was induced only 2.2-fold. These data are consistent with previous reports that demonstrate *N. meningitidis* to have intermediate catalase activity in a Δ *oxyR* mutant, and to have high catalase activity in the presence of H₂O₂ as a result of OxyR-mediated activation (Ieva *et al.*, 2008). These data also demonstrate that in *N. gonorrhoeae*, the induction capacity of *katA* in response to H₂O₂ is much lower, which is likely the reason that OxyR has been reported to act primarily as a repressor (Tseng *et al.*, 2003). The fact that these *lacZ* fusion strains were all analysed in gonococcal strain F62 suggests that the difference in *katA* expression and regulation between these two species is due to differences in the promoter sequence rather than differences in the OxyR protein itself.

Though very few sequence differences exist between the gonococcal and meningococcal *katA* upstream regions, we

reasoned that the single base pair difference in the –10 transcription element could be responsible for the observed differences in basal catalase activity (Fig. 2, Table 2). While the *N. gonorrhoeae* –10 element contains a perfect match to the *E. coli* consensus, 5'-TATAAT-3', the *N. meningitidis* –10 element contains a G at nucleotide –8 (5'-TATAGT-3'; Fig. 2). In order to determine the effect of nucleotide –8 on *katA* expression, site-directed mutagenesis was used to change this nucleotide from A to G in P_{kat}F62::*lacZ*. Basal expression of P_{kat}F62::*lacZ* A→G was comparable with that of P_{kat}MC58::*lacZ* (Table 2), suggesting that the lower basal catalase activity observed in *N. meningitidis* is primarily due to this single nucleotide difference. Surprisingly, this nucleotide was also responsible for OxyR-induction capacity, as P_{kat}F62::*lacZ* A→G, like P_{kat}MC58::*lacZ*, was found to be highly induced in the presence of 2 mM H₂O₂ (36.7-fold; Table 2). Interestingly, these data demonstrate that the large difference in the pattern of *katA* expression between these two species can primarily be explained by a single nucleotide difference.

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_{lac} promoter, and expression was induced in gonococcal strain F62 Δ *katA* 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

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

Data represent the mean $\pm$ SD in Miller units of 12 determinations.

Promoter fusion to <i>lacZ</i> *	Parental	Δ <i>oxyR</i>	Parental + H ₂ O ₂	Fold repression by OxyR†	Fold activation by OxyR‡
P _{kat} F62	430 $\pm$ 70	7000 $\pm$ 570	960 $\pm$ 64	16.1	2.2
P _{kat} MC58	9 $\pm$ 2	140 $\pm$ 18	410 $\pm$ 62	15.4	43.1
P _{kat} F62 TATAAT→TATAGT	15 $\pm$ 2	220 $\pm$ 27	550 $\pm$ 94	14.4	36.7

*All promoter fusions to the *lacZ* gene were assayed in gonococcal strain F62.

†Fold OxyR-mediated repression measured by the expression level in a Δ *oxyR* strain divided by the basal expression from the parental promoter.

‡Fold OxyR-mediated activation measured by the promoter expression following H₂O₂ treatment divided by the basal expression from the parental promoter.

Fig. 3. Catalase activity and survival of inducible *katA*, *katE* and *katG* strains of F62 $\Delta katA$. Exponential phase cultures were diluted to OD₆₀₀ 0.2 and exposed to the indicated concentration of IPTG. After 90 min, the cultures were diluted back to OD₆₀₀ 0.2. (a) An aliquot of the culture was used for measuring catalase activity. Data are representative of three separate experiments. (b) The remainder of the culture was exposed to 2 mM PN and grown out for 45 min before determination of viability. Results are plotted as survival versus catalase activity. Data points are individual values determined from three repetitions of the experiment. Symbols used: gonococcal *katA* (open and closed diamonds), *E. coli katE* (open and closed circles), *E. coli katG* (open and closed triangles).

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 $\Delta katA$ 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 H₂O₂

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 H₂O₂ in a basic homogeneous solvent system, followed by the removal of unreacted H₂O₂ by treatment with

granular manganese dioxide (MnO₂) (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 ($\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$) (Hughes & Nicklin, 1970).

Unlike the Calbiochem PN solution, PN solutions that were prepared in our laboratory failed to cause significant killing of the gonococcal $\Delta katA$ 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 H₂O₂, killing increased with PN concentration, reaching levels observed with the commercial preparation. When dose-response curves for H₂O₂ 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 H₂O₂, and that these compounds act synergistically to cause cell killing.

Effects of bicarbonate on PN/H₂O₂-mediated killing

Gonococci require carbon dioxide for growth, which is supplied in broth culture in the form of sodium bicarbonate.

Fig. 4. Dose-response of individual and combined PN/H₂O₂. All cultures were grown to exponential phase and diluted to OD₆₀₀ 0.2 in GCK (0.042% NaHCO₃). (a) Cultures were exposed to a range of PN concentrations (0–1.75 mM), with (squares) or without (diamonds) addition of 0.2 mM H₂O₂. (b) Cultures were exposed to a range of H₂O₂ concentrations (0–0.3 mM), with (squares) or without (diamonds) immediate previous addition of 2.0 mM PN. Results are representative of one of three independent trials, all of which generated a similarly shaped curve.

However, carbon dioxide is known to react with both PN and H₂O₂ (Augusto *et al.*, 2002; Richardson *et al.*, 2000). To determine the effect of carbon dioxide on PN/H₂O₂-mediated cell killing, the gonococcal $\Delta katA$ strain was subjected to PN or H₂O₂ 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 H₂O₂ (~1 log and ~2 logs of killing, respectively). There was relatively little increase in sensitivity to H₂O₂ 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/H₂O₂ killing. In turn, this would also suggest that it is unlikely that the bicarbonate radical plays a role in synergistic PN/H₂O₂ killing.

Effect of timing on PN/H₂O₂-mediated killing

We next wished to determine whether there was an effect on PN/H₂O₂-mediated killing when these two agents were added sequentially rather than simultaneously. When PN was added 5 min before H₂O₂ addition, we observed approximately 3 logs of cell killing (Fig. 6). When PN was added 5 min after H₂O₂ 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/

H₂O₂-mediated killing effect, which suggests that PN can influence the sensitivity of the organism to H₂O₂.

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. H₂O₂, 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 NO₂ production (Zhu *et al.*, 1992). A

Fig. 5. Survival of F62 $\Delta katA$ after exposure to PN in the presence or absence of sodium bicarbonate. (a) Cultures were grown to exponential phase in GCK (0.042% NaHCO_3), filtered, diluted to OD_{600} 0.2 in fresh medium containing 0.042% NaHCO_3 , and treated with H_2O_2 and/or PN. (b) Cultures were grown to exponential phase in GCK (0.042% NaHCO_3), filtered, diluted to OD_{600} 0.2 in fresh medium lacking NaHCO_3 , and treated with H_2O_2 and/or PN. All cultures were allowed to grow out for 45 min before plating to determine viability. Abbreviations: NA (no addition), PN (2 mM PN), H (0.2 mM H_2O_2), PN+H (simultaneous 0.2 mM H_2O_2 /2 mM PN treatment). Results are representative of three independent determinations; error bars, SD.

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 NO_2 , 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 CO_2 . The effects of CO_2 on PN toxicity in *Trypanosoma cruzi* demonstrate that the rapid reaction of CO_2 with PN decreases its effective toxicity based on the distance that the PN needs to diffuse (Alvarez *et al.*, 2004). The CO_2 reduces the ability of PN to reach its target, and therefore to exert a toxic effect. Another study showed the protective effect of CO_2 on PN toxicity, by comparing *Helicobacter pylori* cultures exposed to PN with and without urea (Kuwahara *et al.*, 2000). Urease breaks down urea into CO_2 and NH_3 , 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 MnO_2 , we removed excess H_2O_2 , 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 H_2O_2 . This suggests that the

Fig. 6. Survival of F62 Δkat following sequential exposure to PN and H_2O_2 . All cultures were grown to exponential phase and diluted to OD_{600} 0.2 in GCK (0.042 % NaHCO_3). Abbreviations: NA (no addition), H→PN (0.2 mM H_2O_2 addition followed by 2 mM PN addition 5 min later), PN→H (2 mM PN addition followed by 0.2 mM H_2O_2 addition 5 min later), PN+H (simultaneous 0.2 mM H_2O_2 /2 mM PN addition). All cultures were allowed to grow out for 45 min before plating to determine viability. Results are representative of three independent determinations; error bars, SD.

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 ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$). 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 ($\cdot\text{OH}$), 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 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 metallo-proteins. These metal ions subsequently react to form potent ROS that cause lethal DNA damage.

We hypothesize that in the presence of CO_2 , 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 $\Delta katA$ 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 $\cdot\text{OH}$ (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 cluster-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 $\cdot\text{OH}$ 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.

ACKNOWLEDGEMENTS

This study was supported by the National Institute of Allergy and Infectious Disease (Public Health Service grant R21 AI 080912). In addition, we thank Ann Jerse, Uniformed Services University, for her generous gift of a *katA* mutant in gonococcal strain FA1090.

REFERENCES

- Alam, M. S., Zaki, M. H., Yoshitake, J., Akuta, T., Ezaki, T. & Akaike, T. (2006). Involvement of *Salmonella enterica* serovar Typhi RpoS in resistance to NO-mediated host defense against serovar Typhi infection. *Microb Pathog* **40**, 116–125.
- Alcorn, T. M., Zheng, H. Y., Gunther, M. R., Hassett, D. J. & Cohen, M. S. (1994). Variation in hydrogen peroxide sensitivity between different strains of *Neisseria gonorrhoeae* is dependent on factors in addition to catalase activity. *Infect Immun* **62**, 2138–2140.
- Alvarez, B. & Radi, R. (2003). Peroxynitrite reactivity with amino acids and proteins. *Amino Acids* **25**, 295–311.
- Alvarez, M. N., Piacenza, L., Irigoien, F., Peluffo, G. & Radi, R. (2004). Macrophage-derived peroxynitrite diffusion and toxicity to *Trypanosoma cruzi*. *Arch Biochem Biophys* **432**, 222–232.
- Archibald, F. S. & Duong, M. N. (1986). Superoxide dismutase and oxygen toxicity defenses in the genus *Neisseria*. *Infect Immun* **51**, 631–641.
- Augusto, O., Bonini, M. G., Amanso, A. M., Linares, E., Santos, C. C. & De Menezes, S. L. (2002). Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. *Free Radic Biol Med* **32**, 841–859.
- Barth, K. R., Isabella, V. M., Wright, L. F. & Clark, V. L. (2009). Resistance to peroxynitrite in *Neisseria gonorrhoeae*. *Microbiology* **155**, 2532–2545.
- Beckman, J. S. & Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* **271**, C1424–C1437.
- Bienert, G. P., Schjoerring, J. K. & Jahn, T. P. (2006). Membrane transport of hydrogen peroxide. *Biochim Biophys Acta* **1758**, 994–1003.
- Bisaillon, J. G., Dubois, G., Beaudet, R., Sylvestre, M., Charbonneau, R. & Gagnon, M. (1985). Quantitative determination of catalase activity produced by *Neisseria gonorrhoeae*, *Staphylococcus epidermidis*, *Neisseria meningitidis* and other bacterial strains using the Catalasemeter. *Exp Biol* **43**, 225–230.
- Bogdan, C., Röllinghoff, M. & Diefenbach, A. (2000). Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol* **12**, 64–76.
- Brunelli, L., Crow, J. P. & Beckman, J. S. (1995). The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. *Arch Biochem Biophys* **316**, 327–334.
- Burney, S., Caulfield, J. L., Niles, J. C., Wishnok, J. S. & Tannenbaum, S. R. (1999). The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat Res* **424**, 37–49.
- Casey, S. G., Shafer, W. M. & Spitznagel, J. K. (1986). *Neisseria gonorrhoeae* survive intraleukocytic oxygen-independent antimicrobial capacities of anaerobic and aerobic granulocytes in the presence of pyocin lethal for extracellular gonococci. *Infect Immun* **52**, 384–389.
- Dyet, K. & Moir, J. (2006). Effect of combined oxidative and nitrosative stress on *Neisseria meningitidis*. *Biochem Soc Trans* **34**, 197–199.
- Edwards, J. L. (2010). *Neisseria gonorrhoeae* survival during primary human cervical epithelial cell infection requires nitric oxide and is augmented by progesterone. *Infect Immun* **78**, 1202–1213.
- Edwards, J. L. & Apicella, M. A. (2004). The molecular mechanisms used by *Neisseria gonorrhoeae* to initiate infection differ between men and women. *Clin Microbiol Rev* **17**, 965–981.
- Gebicka, L. & Didik, J. (2009). Catalytic scavenging of peroxynitrite by catalase. *J Inorg Biochem* **103**, 1375–1379.
- Goldstein, S. & Merényi, G. (2008). The chemistry of peroxynitrite: implications for biological activity. *Methods Enzymol* **436**, 49–61.
- Hampton, M. B., Kettle, A. J. & Winterbourn, C. C. (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**, 3007–3017.

- Heaselgrave, W., Andrew, P. W. & Kilvington, S. (2010). Acidified nitrite enhances hydrogen peroxide disinfection of *Acanthamoeba*, bacteria and fungi. *J Antimicrob Chemother* **65**, 1207–1214.
- Hughes, M. N. & Nicklin, H. G. (1970). The chemistry of peroxonitrites. Part II. Copper (II)-catalysed reaction between hydroxylamine and peroxonitrite in alkali. *J Chem Soc* 925–928.
- Ieva, R., Roncarati, D., Metruccio, M. M., Seib, K. L., Scarlato, V. & Delany, I. (2008). OxyR tightly regulates catalase expression in *Neisseria meningitidis* through both repression and activation mechanisms. *Mol Microbiol* **70**, 1152–1165.
- Inoue, S. & Kawanishi, S. (1987). Hydroxyl radical production and human DNA damage induced by ferric nitrilotriacetate and hydrogen peroxide. *Cancer Res* **47**, 6522–6527.
- Kellogg, D. S., Jr, Peacock, W. L., Jr, Deacon, W. E., Brown, L. & Pirkle, D. I. (1963). *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J Bacteriol* **85**, 1274–1279.
- Kono, Y., Shibata, H., Adachi, K. & Tanaka, K. (1994). Lactate-dependent killing of *Escherichia coli* by nitrite plus hydrogen peroxide: a possible role of nitrogen dioxide. *Arch Biochem Biophys* **311**, 153–159.
- Kuwahara, H., Miyamoto, Y., Akaike, T., Kubota, T., Sawa, T., Okamoto, S. & Maeda, H. (2000). *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. *Infect Immun* **68**, 4378–4383.
- McLean, S., Bowman, L. A. & Poole, R. K. (2010). KatG from *Salmonella typhimurium* is a peroxynitritase. *FEBS Lett* **584**, 1628–1632.
- Mehr, I. J. & Seifert, H. S. (1998). Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. *Mol Microbiol* **30**, 697–710.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Pacelli, R., Wink, D. A., Cook, J. A., Krishna, M. C., DeGraff, W., Friedman, N., Tsokos, M., Samuni, A. & Mitchell, J. B. (1995). Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. *J Exp Med* **182**, 1469–1479.
- Pacher, P., Beckman, J. S. & Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* **87**, 315–424.
- Potter, A. J., Kidd, S. P., Edwards, J. L., Falsetta, M. L., Apicella, M. A., Jennings, M. P. & McEwan, A. G. (2009a). Thioredoxin reductase is essential for protection of *Neisseria gonorrhoeae* against killing by nitric oxide and for bacterial growth during interaction with cervical epithelial cells. *J Infect Dis* **199**, 227–235.
- Potter, A. J., Kidd, S. P., Edwards, J. L., Falsetta, M. L., Apicella, M. A., Jennings, M. P. & McEwan, A. G. (2009b). Esterase D is essential for protection of *Neisseria gonorrhoeae* against nitrosative stress and for bacterial growth during interaction with cervical epithelial cells. *J Infect Dis* **200**, 273–278.
- Prinz, W. A., Aslund, F., Holmgren, A. & Beckwith, J. (1997). The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. *J Biol Chem* **272**, 15661–15667.
- Radi, R., Beckman, J. S., Bush, K. M. & Freeman, B. A. (1991). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* **288**, 481–487.
- Richardson, D. E., Yao, H. R., Frank, K. M. & Bennett, D. A. (2000). Equilibria, kinetics, and mechanism in the bicarbonate activation of hydrogen peroxide: oxidation of sulfides by peroxymonocarbonate. *J Am Chem Soc* **122**, 1729–1739.
- Sahoo, R., Bhattacharjee, A., Majumdar, U., Ray, S. S., Dutta, T. & Ghosh, S. (2009). A novel role of catalase in detoxification of peroxynitrite in *S. cerevisiae*. *Biochem Biophys Res Commun* **385**, 507–511.
- Schmidt, K., Pfeiffer, S. & Mayer, B. (1998). Reaction of peroxynitrite with HEPES or MOPS results in the formation of nitric oxide donors. *Free Radic Biol Med* **24**, 859–862.
- Seib, K. L., Jennings, M. P. & McEwan, A. G. (2003). A Sco homologue plays a role in defence against oxidative stress in pathogenic *Neisseria*. *FEBS Lett* **546**, 411–415.
- Seib, K. L., Tseng, H. J., McEwan, A. G., Apicella, M. A. & Jennings, M. P. (2004). Defenses against oxidative stress in *Neisseria gonorrhoeae* and *Neisseria meningitidis*: distinctive systems for different lifestyles. *J Infect Dis* **190**, 136–147.
- Seib, K. L., Wu, H. J., Kidd, S. P., Apicella, M. A., Jennings, M. P. & McEwan, A. G. (2006). Defenses against oxidative stress in *Neisseria gonorrhoeae*: a system tailored for a challenging environment. *Microbiol Mol Biol Rev* **70**, 344–361.
- Silver, L. E. & Clark, V. L. (1995). Construction of a translational *lacZ* fusion system to study gene regulation in *Neisseria gonorrhoeae*. *Gene* **166**, 101–104.
- Simons, M. P., Nauseef, W. M. & Apicella, M. A. (2005). Interactions of *Neisseria gonorrhoeae* with adherent polymorphonuclear leukocytes. *Infect Immun* **73**, 1971–1977.
- Soler-García, A. A. & Jerse, A. E. (2004). A *Neisseria gonorrhoeae* catalase mutant is more sensitive to hydrogen peroxide and paraquat, an inducer of toxic oxygen radicals. *Microb Pathog* **37**, 55–63.
- Stohl, E. A., Criss, A. K. & Seifert, H. S. (2005). The transcriptome response of *Neisseria gonorrhoeae* to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. *Mol Microbiol* **58**, 520–532.
- Tseng, H. J., McEwan, A. G., Apicella, M. A. & Jennings, M. P. (2003). OxyR acts as a repressor of catalase expression in *Neisseria gonorrhoeae*. *Infect Immun* **71**, 550–556.
- Uppu, R. M. (2006). Synthesis of peroxynitrite using isoamyl nitrite and hydrogen peroxide in a homogeneous solvent system. *Anal Biochem* **354**, 165–168.
- Uppu, R. M. & Pryor, W. A. (1996). Biphasic synthesis of high concentrations of peroxynitrite using water-insoluble alkyl nitrite and hydrogen peroxide. *Methods Enzymol* **269**, 322–329.
- Wengenack, N. L., Jensen, M. P., Rusnak, F. & Stern, M. K. (1999). *Mycobacterium tuberculosis* KatG is a peroxynitritase. *Biochem Biophys Res Commun* **256**, 485–487.
- Yu, K., Mitchell, C., Xing, Y., Magliozzo, R. S., Bloom, B. R. & Chan, J. (1999). Toxicity of nitrogen oxides and related oxidants on mycobacteria: *M. tuberculosis* is resistant to peroxynitrite anion. *Tuber Lung Dis* **79**, 191–198.
- Zhang, Y. & Hogg, N. (2004). Formation and stability of S-nitrosothiols in RAW 264.7 cells. *Am J Physiol Lung Cell Mol Physiol* **287**, L467–L474.
- Zheng, M., Aslund, F. & Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279**, 1718–1722.
- Zhu, L., Gunn, C. & Beckman, J. S. (1992). Bactericidal activity of peroxynitrite. *Arch Biochem Biophys* **298**, 452–457.

Edited by: J. Moir