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Resistance to peroxynitrite in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae encodes a number of important genes that aid in survival during times of oxidative stress. The same immune cells capable of oxygen-dependent killing mechanisms also have the capacity to generate reactive nitrogen species (RNS) that may function antimicrobially. F62 and eight additional gonococcal strains displayed a high level of resistance to peroxynitrite, while *Neisseria meningitidis* and *Escherichia coli* showed a four- to seven-log and a four-log decrease in viability, respectively. Mutation of gonococcal orthologues that are known or suspected to be involved in RNS defence in other bacteria (*ahpC, dnrN* and *msrA*) resulted in no loss of viability, suggesting that *N. gonorrhoeae* has a novel mechanism of resistance to peroxynitrite. Whole-cell extracts of F62 prevented the oxidation of dihydrorhodamine, and decomposition of peroxynitrite was not dependent on *ahpC, dnrN* or *msrA*. F62 grown in co-culture with *E. coli* strain DH10B was shown to protect *E. coli* viability 10-fold. Also, peroxynitrite treatment of F62 did not result in accumulation of nitrated proteins, suggesting that an active peroxynitrite reductase is responsible for peroxynitrite decomposition rather than a protein sink for amino acid modification.

INTRODUCTION

Neisseria gonorrhoeae is a Gram-negative diplococcus and the causative agent of the sexually transmitted infection gonorrhoea. The US Centers for Disease Control (CDC) estimates that nearly 700 000 new cases of gonorrhoea occur each year in the USA, with a total of 65 million cases worldwide (www.cdc.gov). As an obligate human pathogen, the gonococcus is armed with a wide array of mechanisms that impede an appropriate immune response. Prolonged infection may allow bacterial spread from the genitourinary tract, leading to complications such as disseminated gonococcal infection (DGI) and pelvic inflammatory disease (PID) (Edwards & Apicella, 2004).

Efficient clearance of a bacterial infection often relies on the ability of the immune system to generate a variety of reactive oxygen and nitrogen species (ROS and RNS, respectively) (Bogdan *et al.*, 2000). Monocytes, macrophages and neutrophils contain the NADPH phagocyte oxidase complex (PHOX), which is responsible for the generation of $O_2^{\bullet-}$, which either spontaneously or enzymically is disproportionated to H_2O_2, or to hydroxyl radicals ($OH^{\bullet}$), by an iron-catalysed Haber–Weiss reaction (Bogdan *et al.*, 2000). Proinflammatory cytokines produced in response to bacterial infection lead to activation of the inducible nitric oxide synthase (iNOS), resulting in *de novo* iNOS synthesis and micromolar production of nitric oxide (NO) (MacMicking *et al.*, 1997). NO plays an important role in immunity. Through activation or inhibition of key receptors, enzymes and transcription factors, NO is capable of modulating the host immune response (Aktan, 2004; Bogdan, 2001). The NF-κB and mitogen-activated protein kinase pathways have both been shown to be controlled by NO (Pacher *et al.*, 2007).

During the various stages of gonococcal infection there is an onslaught of both oxidative and nitrosative stress (Carreras *et al.*, 1994; Hampton *et al.*, 1998). Previous research has shown that multiple gonococcal gene products aid in survival under oxidative conditions, and $O_2^{\bullet-}$ and H_2O_2 are largely ineffective at eliminating gonococcal infection (Alcorn *et al.*, 1994; Archibald & Duong, 1986; Fu *et al.*, 1989; Rest *et al.*, 1982; Seib *et al.*, 2003). Furthermore, *in vitro* studies have demonstrated that a subpopulation of *N. gonorrhoeae* survive within neutrophils, leukocytes that are armed with both ROS and RNS generation potential (Casey *et al.*, 1979, 1986; Simons *et al.*, 2005). This suggests that the organism is capable of persistent colonization within an activated immune system, and that oxygen-dependent killing mechanisms may not be completely effective at eradicating infection (Seib *et al.*, 2006).

Abbreviations: DETA/NO, 2,2′-(hydroxynitrosohydrazono) bis-ethanimine; DHR, dihydrorhodamine; iNOS, inducible nitric oxide synthase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

A supplementary table, listing bacterial strains used in this study, with accompanying supplementary references, is available with the online version of this paper.

Pathogenic *Neisseria* spp. are capable of anaerobic growth using nitrite or NO as an alternative electron acceptor, and previous studies have examined the toxicity of NO within gonococcal and meningococcal strains (Dyet & Moir, 2006; Householder *et al.*, 2000). Interestingly, a gonococcal strain incapable of reducing NO due to mutation of the nitric oxide reductase gene *norB*, which normally enzymically reduces NO to N_2O, remains viable in the presence of nitrite anaerobically (Householder *et al.*, 2000). This result was unexpected, as *norB* mutations in other denitrifying bacteria have been shown to be lethal under similar conditions (Braun & Zumft, 1991; Cramm *et al.*, 1997).

The simultaneous presence of both $O_2^{\bullet-}$ and NO allows for the generation of peroxynitrite ($ONOO^-$), an RNS that is a stronger oxidant (Goldstein & Merenyi, 2008; Szabo *et al.*, 2007) and is much more reactive than its parent molecules (Beckman & Koppenol, 1996). Peroxynitrite reactivity is highly pH-dependent, and both peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) can participate in one- and two-electron oxidation reactions (Beckman & Koppenol, 1996). Reactivity occurs through two distinct pathways, either direct (oxidative processes by peroxynitrite itself) or indirect (reactions initiated by the radicals formed via decomposition of peroxynitrite) mechanisms (Alvarez & Radi, 2003; Szabo *et al.*, 2007). Reactivity with carbon dioxide leads to the formation of carbonate ($CO_3^{\bullet-}$) and nitrogen dioxide ($NO_2^{\bullet}$) radicals, which are one-electron oxidants. Other reactions can result in peroxynitrite decomposition to nitrite (NO_2^-) and nitrate (NO_3^-) and sulphenic acid derivatives. Alternatively, ONOOH decomposes to hydroxyl ($OH^{\bullet}$) and $NO_2^{\bullet}$ radicals. Peroxynitrite and its decomposition products are capable of oxidizing lipids (Radi *et al.*, 1991b) and DNA (Burney *et al.*, 1999), as well as modifying specific amino acid residues within peptide chains (Pacher *et al.*, 2007). Protein modifications may include oxidation of thiol groups (Radi *et al.*, 1991a) and nitration of tyrosine residues (Reiter *et al.*, 2000).

Peroxynitrite-mediated killing has been shown in a number of bacterial species (De Groote *et al.*, 1995; Zhu *et al.*, 1992). Incubation of *Salmonella enterica* serovars Typhimurium and Typhi with 21 μM $ONOO^-$ results in a six-log loss of viability (Alam *et al.*, 2006). *Escherichia coli* and *Helicobacter pylori* have shown three- and four-log decreases in viability, respectively, when incubated with either molecular-generated $ONOO^-$ or directly added 1 mM $ONOO^-$ (Dyet & Moir, 2006; Kuwahara *et al.*, 2000). Currently, only *Mycobacterium tuberculosis* has shown high levels of peroxynitrite resistance, whereby treatment at a 1 mM concentration results in nearly 100 % viability and is dependent on the strain being virulent (Yu *et al.*, 1999).

Currently, the evaluation of peroxynitrite-mediated lethality in the gonococcus has been overlooked. Here we show that *N. gonorrhoeae* is highly resistant to peroxynitrite, while *E. coli* DH10B and *Neisseria meningitidis* strains show a significant degree of killing. Resistance to peroxynitrite-mediated killing occurs both when it is generated molecularly and when it is added directly to growing cultures. Insertional inactivation of gonococcal orthologues to genes known or suspected to be involved in RNS defence in other bacterial species resulted in wild-type levels of peroxynitrite resistance. This suggests that *N. gonorrhoeae* contains a novel mechanism for avoiding the bactericidal effects of peroxynitrite.

METHODS

Bacterial strains and growth conditions. All gonococcal mutant strains were derived from laboratory strain F62. Other isolates were obtained from the Neisseria Reference Laboratory, CDC, Atlanta, GA. *N. gonorrhoeae* and *N. meningitidis* strains (Supplementary Table S1) were grown on Difco GC medium base (Becton Dickinson) plates with 1 % Kellogg's supplement (GCK) (Kellogg *et al.*, 1963), aerobically in a 5 % CO_2 incubator at 37 °C. For broth cultures, inoculum was taken from overnight-grown bacteria from GCK plates and added to GCK broth plus 0.042 % (w/v) $NaHCO_3$ with shaking at 240 r.p.m. in a Gyrotory water bath shaker (New Brunswick Scientific) at 37 °C. *E. coli* DH10B was the typical cloning strain, grown on either GCK or Luria agar plates at 37 °C. Broth cultures were either in GCK or in Luria broth (LB) plus 0.042 % (w/v) $NaHCO_3$.

Chemicals and reagents. The long-term NO donor DETA/NO [2,2′-(hydroxynitrosohydrazono) bis-ethanimine], hypoxanthine and xanthine oxidase (Sigma) were used in these studies.

Insertional inactivation of genes potentially involved in ROS and RNS defence. Gonococcal strain F62 was used to construct an *aniA* mutant strain with an erythromycin antibiotic cassette inserted within the *aniA* coding region, as previously described (Householder *et al.*, 1999). Similarly, the *norB* coding region was interrupted by an erythromycin antibiotic cassette, as previously described (Householder *et al.*, 2000). Gonococcal strain F62 was used to construct mutant strains that had *ahpC*, *cycP*, *dnrN*, *gor*, *msrA* or NG1184 insertionally inactivated. Each mutant strain had an antibiotic-resistance cassette inserted within the coding region of the respective gene, allowing for genetic screening of the appropriate clones.

Detailed strain construction was performed as follows. *ahpC* was insertionally inactivated by inserting a chloramphenicol-resistance cassette into the gene (NG0328). NG0328 is annotated within the STDGEN gonococcal database (http://stdgen.northwestern.edu/) as bacterioferritin comigratory protein (bcp). A Pfam search examining the protein domains placed NG0328 within the AhpC-TSA family of proteins, in agreement with experimental evidence (Jeong *et al.*, 2000). Consequently we consider NG0328 to be *ahpC*. Two fragments were amplified using GC-Rich *Taq* (Roche). The 5′ fragment began 433 bp upstream from the *ahpC* start site and included 125 bp of the coding region, with a *Pst*I restriction site on the 3′ end. The second fragment had an *Xho*I restriction site on the 5′ end, began 380 bp into the gene and ended 384 bp after the TGA stop codon. The two fragments were cut with either *Pst*I or *Xho*I and ligated with a complementarily digested *cat* cassette, then transformed into strain F62, creating strain RUG 8000.

The cytochrome *c*′ gene (*cycP*) was inactivated by inserting a kanamycin (*aph*) resistance cassette into the gene (NG1080). Two fragments were amplified using AmpliTaq (Applied Biosystems). The 5′ fragment began 744 bp upstream of the *cycP* start site and included

28 bp of the coding region, with an *Xho*I restriction site on the 3′ end. The second fragment had a *Hin*dIII restriction site on the 5′ end, began 445 bp into the gene and ended 793 bp after the TAA stop codon. The two fragments were cut with either *Xho*I or *Hin*dIII and ligated with a complementarily digested *aph* cassette, then transformed into strain F62, creating strain RUG 8008.

The *dnrN* gene was inactivated by inserting an erythromycin-resistance (*erm*) cassette into the gene (NG0653). Two fragments were amplified using GC-rich *Taq*. The 5′ fragment began 500 bp upstream from the *dnrN* start site and included 151 bp of the coding region, with a *Hin*dIII restriction site at the 3′ end. The second fragment had an *Xho*I restriction site on the 5′ end, began 356 bp into the gene and ended 386 bp after the TAA stop codon. The two fragments were cut with either *Hin*dIII or *Xho*I and ligated to a complementarily digested *erm* cassette, then transformed into strain F62, creating strain RUG 8001.

The *msrA* gene was inactivated by inserting a kanamycin (*aph*) resistance cassette into the gene (NG2059). Two fragments were amplified using GC-rich *Taq*. The 5′ fragment began 317 bp upstream from the *msrA* start site and included 299 bp of the coding region, with a *Hin*dIII restriction site at the 3′ end. The second fragment had an *Xho*I restriction site on the 5′ end, began 1320 bp into the gene and ended 471 bp after the TGA stop codon. The two fragments were cut with either *Hin*dIII or *Xho*I and ligated to a complementarily digested *aph* cassette, then transformed into strain F62, creating strain RUG 8002.

The *gor* gene was inactivated by inserting an erythromycin-resistance cassette into the gene (NG0925). Two fragments were amplified using AmpliTaq. The 5′ fragment began 692 bp upstream from the *gor* start site and included 237 bp of the coding region, with a *Pst*I restriction site at the 3′ end. The second fragment had an *Xho*I restriction site on the 5′ end, and began 88 bp upstream of the TGA stop codon. The two fragments were cut with *Pst*I or *Xho*I and ligated to a complementarily digested *erm* cassette, then transformed into strain F62, creating strain RUG 8010.

NG1184 was inactivated by inserting an erythromycin-resistance cassette into the gene, creating strain RUG 8011. Two fragments were amplified using AmpliTaq. The 5′ fragment began 680 bp upstream from the transcriptional start site and included 263 bp of the coding region, with a *Pst*I restriction site at the 3′ end. The second fragment had an *Xho*I restriction site on the 5′ end, began 1062 bp into the gene and ended 851 bp after the TAA stop codon. The two fragments were cut with either *Pst*I or *Xho*I and ligated to a complementarily digested *erm* cassette, then transformed into strain F62. RUG 8012 was created by transforming this same DNA fragment into F62.

Strains that contained two or three insertionally inactivated genes (RUG 8003, RUG 8004, RUG 8005, RUG 8006, RUG 8012) were created using the same methods, except that the same strain was used for each transformation, thus generating multiple gene inactivations within the same genetic background. Cloning of DNA sequences was verified at ACGT, Inc.

Survival counts of bacteria exposed to RNS produced by molecular generation. Gonococcal strain F62 and *E. coli* strain DH10B were grown in GCK and LB respectively, in a water bath shaker to OD_{600} 0.5 and diluted to OD_{600} 0.25 into fresh media containing a final concentration of either 2 mM DETA/NO, 0.1 units xanthine oxidase ml^{-1} and 250 μM hypoxanthine, or 2 mM DETA/NO and 0.1 units xanthine oxidase ml^{-1} and 250 μM hypoxanthine. At time points of 0, 30, 60, 90 and 120 min, bacteria were diluted and plated to determine viability. The extent of RNS-mediated killing or growth of the culture was calculated by dividing the measured c.f.u. by the initial c.f.u.

Survival counts with direct peroxynitrite addition. The ability of peroxynitrite to kill various gonococcal and meningococcal strains, as well as *E. coli* DH10B, was tested by directly adding peroxynitrite (Calbiochem) at final concentrations of 1 or 2 mM, or no addition, to exponential-phase cultures diluted into fresh GCK broth plus $NaHCO_3$. Peroxynitrite is stable in 4.7 % NaOH and begins to decompose at pH 8 or below. Peroxynitrite-mediated killing was examined by diluting bacteria and plating at time points of 0, 30, 60, 90 and 120 min to determine cell viability as described above. For experiments determining whether *N. gonorrhoeae* could protect *E. coli* from RNS-mediated killing, F62 and *E. coli* DH10B were added to cultures at an input ratio of either 1 : 1 or 5 : 1 with respect to OD_{600}. *E. coli* DH10B was added at a constant OD_{600} of 0.125 for all conditions, while F62 was added at an OD_{600} of either 0.125 or 0.625. *E. coli* viable cell counts were obtained by dilution-plating of cultures with either no addition or addition of 1 mM peroxynitrite after 120 min of growth on Luria agar plates. This medium supports growth of *E. coli* but not *N. gonorrhoeae*.

The dose response of peroxynitrite-mediated killing was assayed by following the viability of strains when exposed to increasing concentrations of peroxynitrite, ranging from no addition to 1 mM. After 120 min, the cells were diluted and plated to determine viability. The c.f.u. at 120 min was divided by the initial c.f.u.

Prevention of dihydrorhodamine (DHR) oxidation by whole bacterial cells. To assay for apparent peroxynitrite reductase activity, cells were tested for their ability to block peroxynitrite-mediated oxidation of DHR (Sigma). Gonococcal strains and *E. coli* DH10B were grown to OD_{600} 1.0. The cells were then washed and resuspended in a solution of DHR (50 μM) in 0.1 M phosphate buffer, pH 7.3, containing 0.1 mM diethylenetriamine-pentaacetic acid and 5 mM glucose. The cells were concentrated 10-fold to increase the probability of cells reacting with peroxynitrite before it reacted with DHR. At time 0, peroxynitrite in 4.7 % NaOH was added to a final concentration of 100 μM under conditions of intense stirring. Conversion of DHR to rhodamine was determined spectrophotometrically (ε=78 800 $M^{-1}cm^{-1}$), with the background and final absorbance at 500 nm determined before and 1 min after peroxynitrite addition.

SDS-PAGE and Western blotting for protein nitration. A control reaction of purified BSA (Promega) at 5 mg ml^{-1}, or bacterial cultures grown to OD_{600} 0.5 in GCK broth plus $NaHCO_3$ and diluted to OD_{600} 0.25, were exposed to three treatments with peroxynitrite to a final concentration of 1 mM with continuous mixing. Bacterial cultures were concentrated by spinning down 1 ml cells and resuspending in 50 μl Tris-buffered saline prior to peroxynitrite addition, or by harvesting cells at 5 and 30 min following the outgrowth stage while in a water shaker bath after peroxynitrite treatment. Samples of purified BSA were taken prior to and after peroxynitrite addition. The cells were lysed with SDS sample buffer lacking DTT and the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-nitrotyrosine mAb (clone 1AG, Upstate Biotechnology). A sheep anti-mouse IgG horseradish peroxidase-linked antibody was used for secondary probing. Antibody binding was detected by ECL (Amersham Pharmacia Biotech).

RESULTS

Resistance to RNS and ROS

We examined the effects of oxidative and nitrosative stress on the growth and viability of gonococcal strain F62 by

adding molecular generators to growing cultures that produced $O_2^{\bullet-}$ (xanthine oxidase), NO (DETA/NO; long-term NO donor) or a combination of both to produce peroxynitrite (De Groote *et al.*, 1997). It should be noted that the xanthine oxidase/hypoxanthine enzymic system generates equimolar amounts of $O_2^{\bullet-}$ and H_2O_2; however, catalase activity is constitutive and is nearly 100 times higher in *N. gonorrhoeae* than in *E. coli* (Hassett *et al.*, 1990). Gonococci grew normally in the presence of DETA/NO (Fig. 1a). In the presence of xanthine oxidase, gonococcal growth was arrested for 60 min, after which time growth resumed normally. Surprisingly, gonococci still survived in the presence of both the NO and $O_2^{\bullet-}$ generator, though growth was inhibited. It is important to note that F62 showed no reduction in viability under any condition. Conversely, *E. coli* DH10B showed 10-fold reduced viability in the presence of $O_2^{\bullet-}$ and 100-fold reduced viability under conditions of $ONOO^-$ generation (Fig. 1b).

Resistance of *N. gonorrhoeae* but not *E. coli* to peroxynitrite

The use of molecular generators to produce peroxynitrite raises concerns because it is not possible to determine the effects of each reactive species within the culture (NO, $O_2^{\bullet-}$ and $ONOO^-$). To address this, experiments were modified to use the direct addition of peroxynitrite. As seen in Fig. 2(a), *N. gonorrhoeae* displayed significant resistance to the direct addition of peroxynitrite over a range of 1 μM to 1 mM. Even at the highest concentration of RNS, there was no loss in gonococcal viability. Conversely, *E. coli* was highly susceptible to RNS-mediated killing, displaying an approximate four-log decrease in viability at a concentration of 1 mM $ONOO^-$ (Fig. 2a).

Growth of *N. gonorrhoeae* with peroxynitrite treatment

We next monitored the viability of F62 over time after direct addition of 1 mM $ONOO^-$ to growing cultures. F62 not only maintained cell viability, but was actually capable of growth over a 120 min time period (Fig. 2b). These data show that *N. gonorrhoeae* is highly resistant to peroxynitrite. In contrast to this finding, *E. coli* viability was significantly affected, with a decrease of three to four logs. Since peroxynitrite has an extremely short half-life of approximately 1 s, the initial burst of peroxynitrite presumably results in killing of *E. coli*, while it appears to only cause growth arrest in *N. gonorrhoeae*. When the RNS is no longer present, growth resumes after a period of time, dependent on peroxynitrite concentration. This suggests that the gonococcus has a mechanism either to escape the RNS or to repair RNS damage, rather than to adapt to RNS addition.

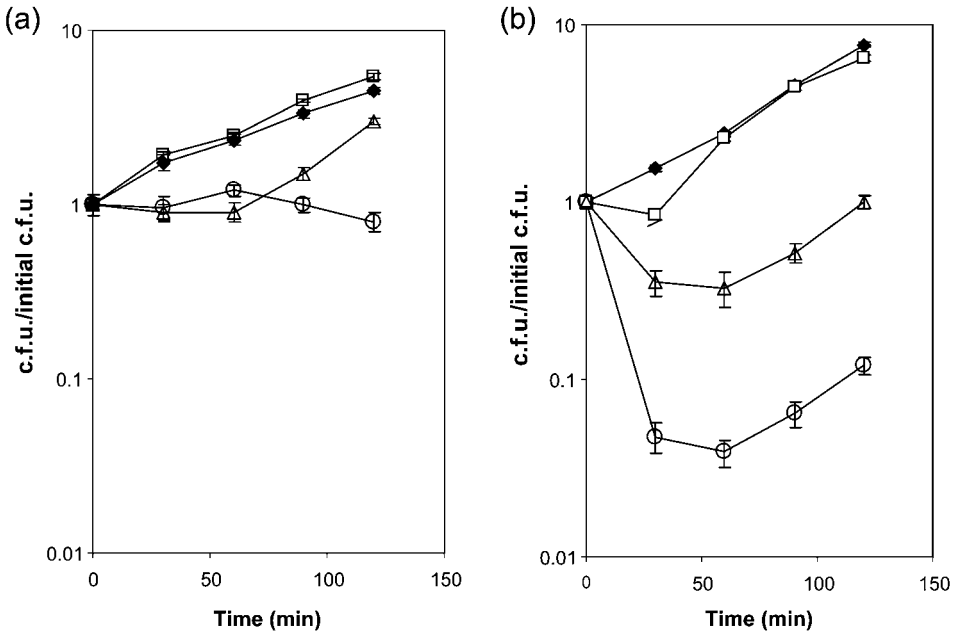

Fig. 1. Effects of ROS and RNS on *N. gonorrhoeae* F62 (a) and *E. coli* DH10B (b) viability. Exponential-phase bacterial cultures were diluted to OD_{600} 0.25 into fresh media containing the following final concentrations: no addition (◆); 2 mM DETA/NO (□); 0.1 units xanthine oxidase ml^{-1} and 250 μM hypoxanthine (△); 2 mM DETA/NO, 0.1 units xanthine oxidase ml^{-1} and 250 μM hypoxanthine (○). At the indicated times, bacteria were diluted and plated to determine viable counts. Results represent the mean ± SE of three independent determinations.

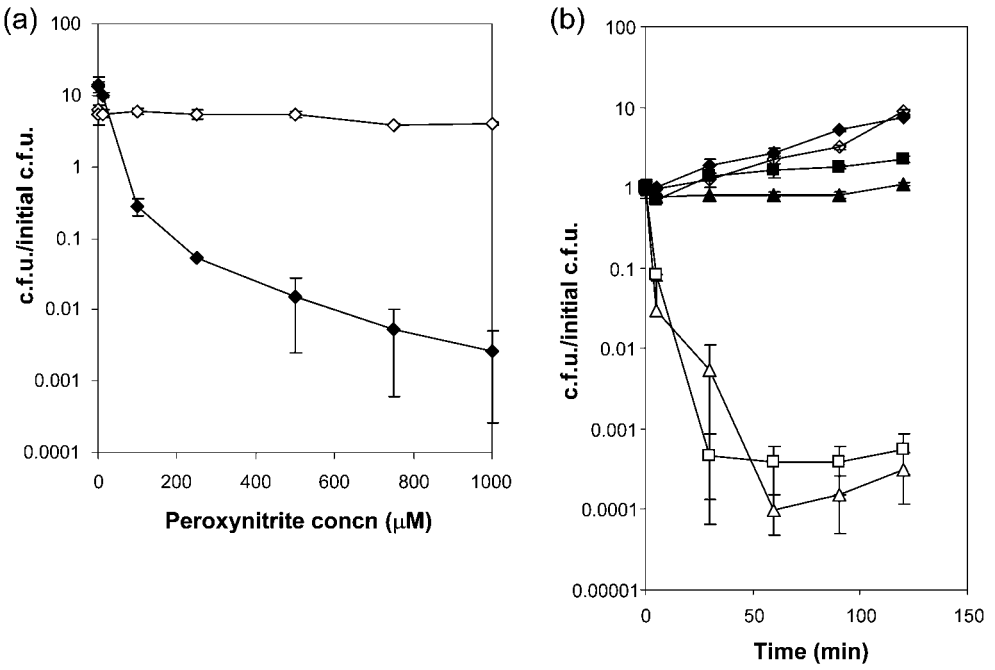

Fig. 2. Dose response and time course of peroxynitrite treatment. (a) Resistance of *N. gonorrhoeae* to direct peroxynitrite addition, while *E. coli* shows a dose response to peroxynitrite-mediated killing. Exponential-phase cultures of gonococcal strain F62 (◇) and *E. coli* DH10B (◆) were diluted into fresh GCK media and the indicated final concentration of peroxynitrite was added at time 0. Following 120 min outgrowth, the cells were diluted and plated for viability. Values represent the mean ± SE of three determinations of the calculated c.f.u./initial c.f.u. (b) Growth of *N. gonorrhoeae* in the presence of high concentrations of peroxynitrite. Exponential-phase gonococcal strain F62 (filled symbols) or *E. coli* DH10B (open symbols) was diluted into fresh GCK medium and peroxynitrite was added at a final concentration of 0 (diamonds), 1 mM (squares) or 2 mM (triangles). At the indicated times, samples were taken to determine viable counts. Values represent the mean ± SE of three determinations of the calculated c.f.u./initial c.f.u.

Peroxynitrite resistance is common to other gonococcal strains

To ensure that the resistance of F62 to $ONOO^-$ was not unique, eight additional gonococcal strains were assayed for $ONOO^-$ resistance. Though there was some period of growth cessation in the presence of the RNS, as seen previously, all eight strains were capable of growth in the presence of $ONOO^-$ (Fig. 3), suggesting that $ONOO^-$ resistance is common in this species. The data for *E. coli* were included for comparison, illustrating the effects of peroxynitrite on the viability of a sensitive organism.

N. meningitidis is highly susceptible to peroxynitrite-mediated killing

Since high levels of $ONOO^-$ resistance were common to a variety of gonococcal strains, we wished to determine whether the other pathogenic *Neisseria* species, *N. meningitidis*, displayed a similar level of resistance. In contrast to what was observed in the gonococcal strains, meningococcal strains that were members of serogroups A, B and C all displayed high levels of peroxynitrite sensitivity (Fig. 4). The effect of peroxynitrite on meningococcal cell viability ranged from a four-log loss in viability (serogroup A) to nearly 100 % cell death, with a loss of viability of six to seven logs (serogroups B and C). This suggests that $ONOO^-$ resistance is not common among *Neisseria* spp.

It has been reported that *N. meningitidis* strain MC58 shows a limited amount of cell death when $O_2^{\bullet-}$ and NO generators are added to cells (Dyet & Moir, 2006). Since peroxynitrite forms from $O_2^{\bullet-}$ and NO, removal of either or both of these species will preclude formation of peroxynitrite. Both $O_2^{\bullet-}$ and NO can be removed from the system by meningococcal superoxide dismutase and nitric oxide reductase, respectively. Consequently, one is unaware of how much if any $ONOO^-$ is produced, especially since the NO donor 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA/NO) has a half-life of only 90 s. Thus, we have evaluated *N. meningitidis* viability during direct $ONOO^-$ addition in order to eliminate any interference by the bacterium in the process of $ONOO^-$ generation. Comparison of peroxynitrite-mediated killing when peroxynitrite was added directly or generated via molecular generators gave significant differences in the viability of *E. coli* (compare Fig. 1b and Fig. 2b).

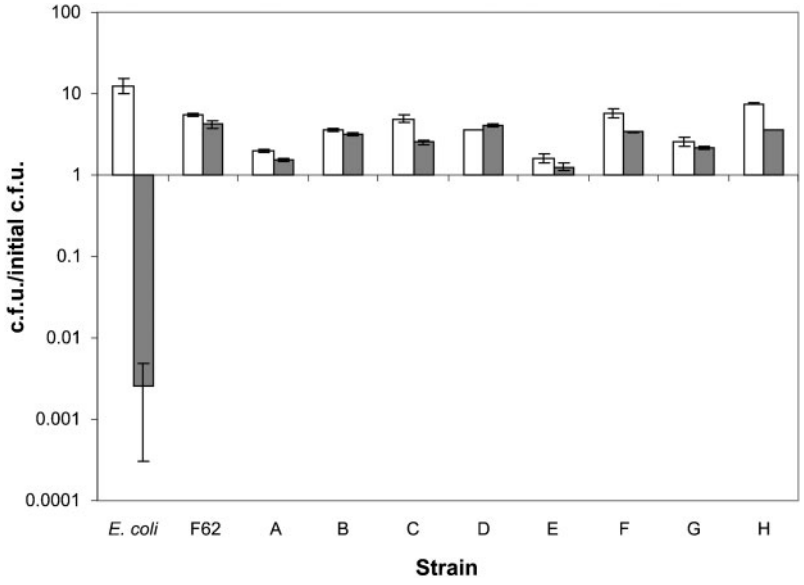

Fig. 3. Resistance of gonococcal strains to peroxynitrite. Exponential-phase bacterial strains were diluted into fresh GCK medium. Bacteria were diluted and plated for viable cell counts at time 0. Peroxynitrite was added at a final concentration of 0 (white bars) or 1 mM (grey bars). Viable counts were determined following 120 min incubation with or without peroxynitrite. Values represent the mean ± SE of the calculated c.f.u./initial c.f.u. of three independent determinations. Strains are: *E. coli* DH10B, *N. gonorrhoeae* F62, NRL 905 (A), RUN 4002 (B), RUN 4383 (C), RUN 5632 (D), RUN 5635 (E), RUN 5636 (F), RUN 5638 (G) and RUN 5640 (H).

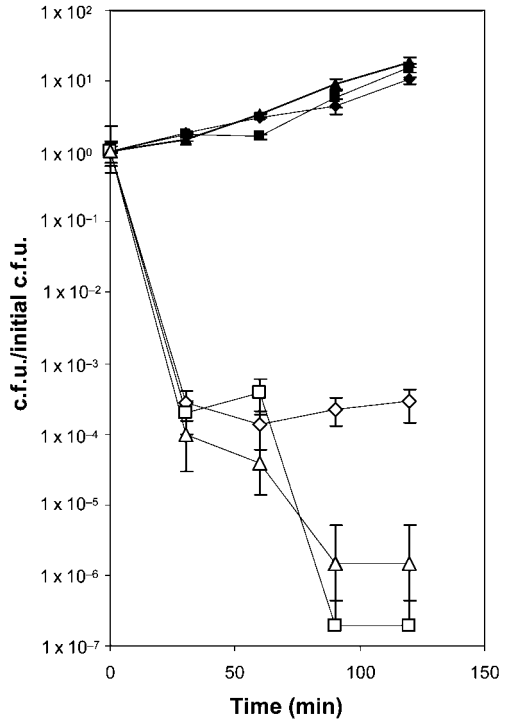

Fig. 4. High-level peroxynitrite-mediated killing in *N. meningitidis* serogroups A, B and C. Exponential-phase *N. meningitidis* strains NRL-9205 (serogroup A; diamonds), NRL-9206 (serogroup B; triangles) and NRL-9207 (serogroup C; squares) were diluted into fresh GCK medium and peroxynitrite was added at a final concentration of 0 (filled symbols) or 1 mM (open symbols). At the indicated times, bacteria were diluted and plated to determine viable counts. The data are the mean ± SE of the calculated c.f.u./initial c.f.u. of three independent determinations.

Effect on resistance to peroxynitrite of mutation in gonococcal genes orthologous to known or putative RNS resistance genes

Resistance to ROS and RNS has been studied in a number of bacterial species, especially members of the *Enterobacteriaceae* and *Mycobacterium* spp. (Alam *et al.*, 2006; Chan *et al.*, 2001; De Groote *et al.*, 1995; Dyet & Moir, 2006; Kuwahara *et al.*, 2000). As a result of this work, a number of ROS/RNS resistance genes have been identified (Table 1). The sequences of these known genes were used to BLAST-search the gonococcal genome for orthologues that may have a similar function. *N. gonorrhoeae* contained genes orthologous to *ahpC* (NG0328), encoding a protein shown to act as an alkyl hydroperoxidase and peroxynitrite reductase, *dnrN* (NG0653), a gene induced by NO and encoding a protein shown to be involved in Fe–S cluster repair following oxidative or nitrosative stress, and *msrA* (NG2059), encoding a protein involved in repair of oxidized methionines (Bryk *et al.*, 2000; Overton *et al.*, 2006, 2008; Poole, 2005; Skaar *et al.*, 2002). Interestingly, the gene for the highly conserved NO-scavenging protein, *hmp*, as well as the genes found to confer resistance to RNS in *M. tuberculosis* (*noxR1*, *noxR3*, *cysH*) (Ehrt *et al.*, 1997; Ruan *et al.*, 1999; Senaratne *et al.*, 2006) and *Mycobacterium marinum* (*mel2*) (Subbian *et al.*, 2007), were not found in the gonococcus.

To determine whether any of these genes function in peroxynitrite resistance, we constructed *ahpC*, *dnrN* and *msrA* mutants and assayed these strains as described above. Mutations in *ahpC*, *dnrN* or *msrA*, alone or in combination, had little effect on peroxynitrite resistance. The addition of increasing concentrations of peroxynitrite extended the period in which the RNS caused growth cessation. All strains eventually recovered and resumed

Table 1. Bacterial genes identified or proposed to be involved in resistance to reactive nitrogen and oxygen species

Gene	Protein	Function	Reactive species	Gonococcal orthologue*	Meningococcal orthologue†
Oxidative stress defences					
ccp	Cytochrome *c* peroxidase	Converts H_2O_2 to O_2	H_2O_2	NG1769	None
cycP	Cytochrome *c′*	Binds NO	NO	NG1080	NMA1119
gor	Glutathione reductase	Maintains glutathione in its reduced form	Indirect defence from ROS	NG0925	NMA1142
kat	Catalase	Converts H_2O_2 to O_2 and H_2O	H_2O_2	NG1767	NMA0050
prx	Peroxiredoxin	Converts H_2O_2 to O_2	H_2O_2	NG0926	NMA1141
sodB	Superoxide dismutase	Converts $O_2^{\bullet-}$ to H_2O_2 (nearly undetectable activity in *N. gonorrhoeae*)	$O_2^{\bullet-}$	NG0405	NMA1104
Reactive nitrogen stress defences					
aniA	Nitrite reductase	Converts NO_2 to NO	NO	NG1276	NMA1887
norB	Nitric oxide reductase	Converts NO to N_2O	NO	NG1275	NMA1886
ahpC	Alkyl hydroperoxide reductase subunit C	Acts as a $ONOO^-$ reductase	$ONOO^-$	NG0328	NMA0963
dnrN	Repair of iron centre (RIC) protein	Expression induced by NO and proposed to be involved in Fe–S cluster repair	Indirect defence from RNS	NG0653	NMA1577
msrA	Peptide methionine sulfoxide reductase	Repairs methionines damaged by $ONOO^-$	Indirect defence from RNS	NG2059	NMA0290
hmp	Flavohaemoglobin (nitric oxide dioxygenase)	Major NO scavenging protein converts NO to either NO_3 or N_2O	NO	None	None
mel2 (*M. marinum*)	Alkanesulfonate monoxygenase	Putative flavin-dependent oxidoreductase; luciferase-like monooxygenase	H_2O_2, NO, $ONOO^-$	None	None
noxR1 (*M. tuberculosis*)	Nitric oxide resistance protein	Confers resistance to RNS when cloned in *E. coli*	NO, $ONOO^-$	None	None
noxR3 (*M. tuberculosis*)	Nitric oxide resistance protein	Confers resistance to RNS when cloned in *E. coli*	NO, $ONOO^-$	None	None
cysH (*M. tuberculosis*)	Phosphoadenosine phosphosulfate reductase	Protects *M. tuberculosis* against $ONOO^-$ and H_2O_2	H_2O_2, $ONOO^-$	None	None

*Annotation number from *N. gonorrhoeae* FA1090 genome on STDGEN database (http://stdgen.northwestern.edu/).

†Annotation number from *N. meningitidis* Z2491 genome on National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/).

growth unless peroxynitrite reached a 2 mM concentration (Fig. 5).

Effect on resistance to peroxynitrite of mutation in gonococcal genes involved in nitric oxide or oxidative stress

We broadened our analysis to examine not only genes involved in nitrosative stress, but also those that are important in oxidative stress, in addition to genes that are involved in NO metabolism. The genes of interest were *aniA* and *norB*, considering their involvement with the RNS nitric oxide. It is not uncommon to have protective proteins show interactions with more than one type of reactive species. The ability of a single protein to be involved in multiple protective systems, such as ROS and RNS defence, is however much more uncommon. Cytochrome *c′* (*cycP*) and cytochrome *c* peroxidase (*ccp*) have been suggested to be involved in defence against oxidative and nitrosative stress (Seib *et al.*, 2004; Stevanin *et al.*, 2005; Turner *et al.*, 2003, 2005). A gonococcal glutathione reductase (*gor*) was examined, since alterations in its function to maintain high levels of reduced glutathione could greatly limit any protective effect of glutathione against peroxynitrite. Gonococcal ORF NG1184 was examined because of its annotation as an NADH oxidoreductase that is completely absent from the meningococcal genome. Lastly, a ΔNG1184 Δ*ahpC* double mutant strain was examined, since the two gene products may have an active role in directly detoxifying peroxynitrite. Mutations in any of the genes examined showed no peroxynitrite-induced killing, although there were slight changes to the amount of growth after peroxynitrite treatment compared with wild-type F62 (Fig. 6). These data suggest that gonococcal resistance to peroxynitrite is the result of redundant systems or a novel gene product.

Peroxynitrite reductase assay

To determine whether gonococcal cells contain peroxynitrite reductase activity, we examined the ability of F62 to block peroxynitrite-mediated oxidation of DHR (Ischiropoulos *et al.*, 1999; Kooy *et al.*, 1994). Both the parental strain F62 and a strain containing mutations in putative RNS resistance genes, F62 Δ*ahpC* Δ*dnrN* Δ*msrA*, effectively prevented the oxidation of DHR (Fig. 7), suggesting that a novel peroxynitrite reductase exists in *N. gonorrhoeae*. Both gonococcal strains also showed an enhanced ability to prevent DHR oxidation compared with *E. coli* DH10B (Fig. 7).

There exists a possibility that other gonococcal components compete with DHR for $ONOO^-$-induced oxidation. To address this concern, we investigated the nitration of proteins as a result of $ONOO^-$ treatment. Gonococcal cells were exposed to three treatments with $ONOO^-$ to a final concentration of 1 mM. The cells were then lysed, and whole-cell extracts were assayed by Western analysis using anti-nitrotyrosine antibody as a probe. There was no detectable nitration of gonococcal proteins under conditions in which BSA was heavily nitrated by peroxynitrite

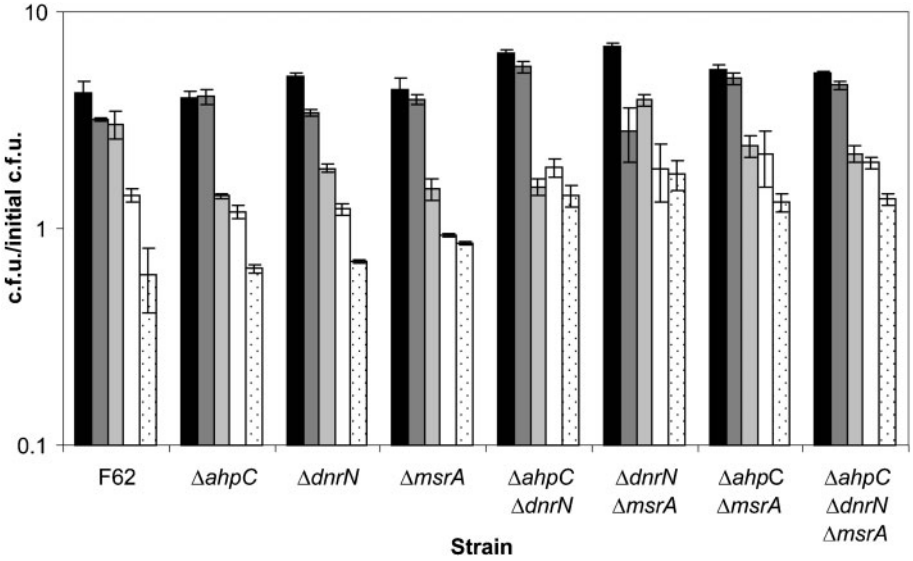

Fig. 5. Mutation of RNS defence genes does not alter the high level of resistance to peroxynitrite. Exponential-phase gonococcal strains were diluted into fresh GCK medium and peroxynitrite was added at a final concentration of 0 (black bars), 0.25 mM (dark grey bars), 1 mM (light grey bars), 1.5 mM (white bars) or 2 mM (stippled bars). Viable counts were determined before RNS addition and after 120 min incubation with peroxynitrite to determine growth. Results are represented as the mean ± SE of the calculated c.f.u./initial c.f.u. of three determinations.

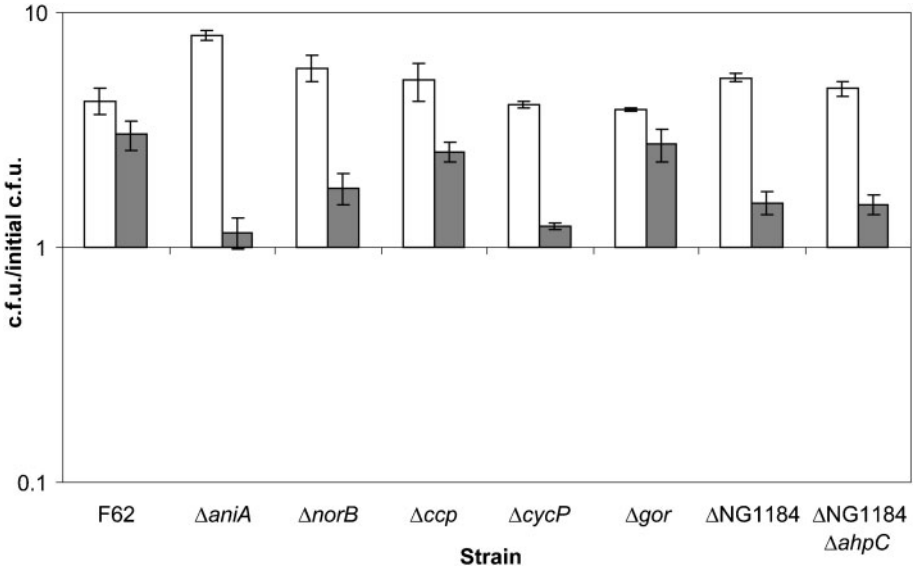

Fig. 6. Gonococcal mutants having inactivated genes involved with nitric oxide and oxidative stress remain resistant to peroxynitrite. Exponential-phase gonococcal strains were diluted into fresh GCK medium. Bacteria were diluted and plated for viable cell counts at time 0. Peroxynitrite was added at a final concentration of 0 (white bars) or 1 mM (grey bars). Viable counts were determined following 120 min incubation with or without peroxynitrite. Values represent the mean ± SE of the calculated c.f.u./initial c.f.u. of three independent determinations.

(data not shown). These results would be expected if peroxynitrite is reduced before protein nitration can occur.

A second approach was used in order to establish that an active mechanism is responsible for peroxynitrite resistance. When *N. gonorrhoeae* was added to a culture of *E. coli* prior to peroxynitrite treatment, there was significant protection from RNS-mediated killing. If *N. gonorrhoeae* was present within the culture at a 1 : 1 ratio with respect to OD_{600}, there was a 10-fold level of protection, while a 5 : 1 ratio (*N. gonorrhoeae* : *E. coli*) caused an increase in *E. coli* viability 100-fold (Fig. 8). This suggests that a peroxynitrite reductase activity is present in exponential-phase *N. gonorrhoeae* cultures, and is capable of preventing peroxynitrite-induced killing in *E. coli.*

DISCUSSION

The importance of ROS in host defence is apparent when the consequences of deficient production are examined. Chronic granulomatous disease (CGD) is a hereditary disease in which patients can have mutations in any of the four required subunits of PHOX (gp91-phox, p47-phox, p22-phox, p67-phox), with the *gp91-phox* gene being the most commonly mutated (Roos *et al.*, 1996). Phagocytes of CGD patients are incapable of ROS generation, and subsequent effects include recurring bouts of infection by *Staphylococcus aureus*, *Klebsiella* spp., *Aspergillus fumigatus* and *Candida albicans* (Fang, 2004).

The production of reactive nitrogen intermediates has only recently become appreciated with respect to its role in host defence. Although there is no identified primary genetic deficiency of iNOS in humans, studies using isolated macrophages or knockout mice have demonstrated an instrumental contribution to pathogen control (Bogdan, 2001). Mice with single deficiencies in iNOS show increased susceptibility to a number of parasitic, bacterial and viral infections, including *Leishmania major* and *M. turberculosis* (Chan *et al.*, 2001; Green *et al.*, 1990). In infections caused by *Listeria monocytogenes*, *Salmonella* spp. and *Chlamydia pneumoniae*, iNOS has been shown to contribute to host defence but not to be essential (Alam *et al.*, 2002; MacMicking *et al.*, 1995; Mayer *et al.*, 1993; Umezawa *et al.*, 1997).

Pathogenic micro-organisms utilize a broad range of strategies to counteract the effects of RNS. Such strategies may include tightly regulated systems of oxidant avoidance or scavenging, as well as the repair of stress-damaged biomolecules (Chakravortty & Hensel, 2003). Detoxification of RNS to less toxic species is a common defensive tactic (Fang, 2004). *S. enterica* serovar Typhimurium utilizes flavohaemoglobin, Hmp, to convert NO to nitrate or nitrous oxide, depending on the

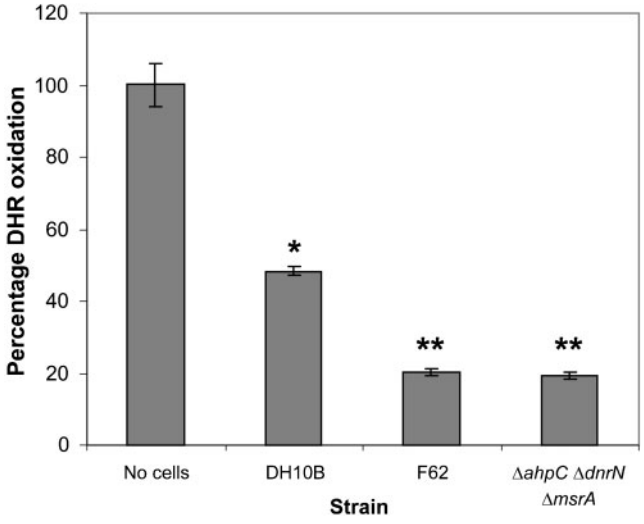

Fig. 7. *N. gonorrhoeae* peroxynitrite reductase activity. Washed cells of gonococcal strain F62, the triple mutant F62 Δ*ahpC* Δ*dnrN* Δ*msrA*, and *E. coli* DH10B were concentrated 10-fold and tested for ability to convert DHR to rhodamine, as described in Methods. Background and final A_{500} were determined before and 1 min after addition of peroxynitrite. Assays were performed in triplicate and the values represent the mean ± SE percentage DHR oxidation. Highly significant differences comparing *E. coli* DH10B with the no-cells control are indicated by an asterisk ($P<0.001$), while significant differences comparing either F62 or F62 Δ*ahpC* Δ*dnrN* Δ*msrA* with *E. coli* DH10B are indicated by double asterisks ($P<0.00005$). Statistical analysis included a Student's *t* test assuming equal variance.

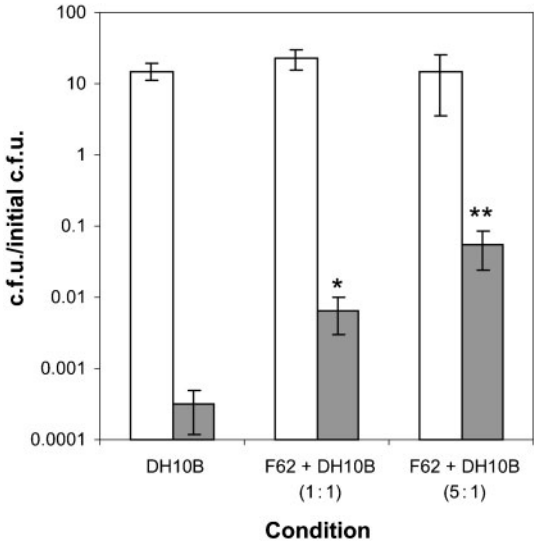

Fig. 8. *N. gonorrhoeae* protects *E. coli* from RNS-mediated killing. F62 and *E. coli* were grown separately in GCK broth then diluted into fresh medium with either *E. coli* DH10B alone, F62 + DH10B (1 : 1 ratio) or F62 + DH10B (5 : 1 ratio). Cells were treated with either no addition (white bars) or 1 mM peroxynitrite (grey bars). After 120 min the cells were diluted and plated for viability. The data are the mean ± SE of three independent determinations. Highly significant differences compared with *E. coli* DH10B culture alone in the presence of 1 mM peroxynitrite are indicated by an asterisk ($P<0.02$) or double asterisks ($P<0.007$). Statistical analysis included a Student's *t* test assuming equal variance.

concentration of oxygen present (Poole & Hughes, 2000). Organisms that contain elements of the denitrification pathway can directly reduce NO to N_2O, e.g. by the nitric oxide reductase in *N. gonorrhoeae* (Cardinale & Clark, 2005; Householder *et al.*, 2000). Alkyl hydroperoxide reductase (*ahpC*) is widespread among bacteria and has been shown to be capable of reducing peroxynitrite to nitrite (Bryk *et al.*, 2000), though loss of this activity did not increase gonococcal sensitivity to peroxynitrite (Fig. 5).

RNS avoidance may also be considered an important survival mechanism. Components capable of eliminating ROS, such as superoxide dismutase (SOD) and catalase (Kat), indirectly lower the potential to generate more damaging nitrogen oxide radicals, including peroxynitrite (Bogdan *et al.*, 2000; Pacher *et al.*, 2007). Gonococci display unusually high catalase and peroxidase activities, while also maintaining very high glutathione levels (Archibald & Duong, 1986). This may account for the inability of oxidative stresses to kill the organism. *Francisella tularensis* utilizes phase variation to alter LPS structure so that it no longer activates iNOS, leading to intracellular replication of the pathogen in rat macrophages (Cowley *et al.*, 1996). *H. pylori* encodes an arginase that degrades the substrate for iNOS activity, L-arginine, preventing NO concentrations from reaching bactericidal levels (Gobert *et al.*, 2001). *S. enterica* is capable of disrupting eukaryotic cell trafficking in such a manner that an insufficient amount of iNOS reaches the mature phagosome. As a result, peroxynitrite has been shown to be absent from vacuoles containing bacteria (Chakravortty *et al.*, 2002). Even without affecting peroxynitrite resistance, gonococcal *cycP* may be important in binding NO *in vivo*, thus preventing peroxynitrite generation (Turner *et al.*, 2005).

In the event that damage occurs as a result of oxidant production, many bacteria encode machinery that repairs damaged biomolecules. Viability can be directly related to the ability to repair DNA damage. The activity of RecBC is essential in *S. enterica*, and strains deficient in RecBC are more susceptible to killing by RNS (Shiloh *et al.*, 1999). A gonococcal *recN* mutant shows increased sensitivity to hydrogen peroxide and polymorphonuclear leukocyte killing (Stohl *et al.*, 2005). Proteins are susceptible to RNS-mediated damage, especially at specific amino acid residues, motifs or cofactors. Such damage includes cysteine oxidation, tyrosine nitration and changes to transition metal centres, all of which can greatly modify protein function and/or activity (Pacher *et al.*, 2007). Methionine sulfoxide reductase (Msr) is widespread

among bacterial species and functions to reduce oxidized methionines, thus preventing excessive accumulation of non-functional proteins (Moskovitz, 2005). *N. gonorrhoeae* has a unique genetic organization, in which *msrA* and *msrB* are translationally fused to form a single polypeptide, unlike *E. coli*, which has two distinct transcription units (Ezraty *et al.*, 2005). However, we have shown that *msrA* is dispensable for peroxynitrite resistance in the gonococcus (Fig. 5).

The search for the gene products responsible for the high levels of gonococcal peroxynitrite resistance has eliminated *ahpC*, *ccp*, *cycP*, *dnrN*, *msrA* and NG1184. The gonococcus also encodes a peroxiredoxin (*prx*), having 98 % sequence identity to Prx of *N. meningitidis*, which is able to reduce H_2O_2 in the presence of GSH (Rouhier & Jacquot, 2003). It is unlikely that Prx is involved in detoxification of peroxynitrite, since it has been identified as a member of the OxyR regulon, in which its expression is upregulated in the presence of H_2O_2 (Seib *et al.*, 2007), comprising a component of oxidative stress defence. Since peroxynitrite has such a short half-life, our experimental setup prevents the involvement of those protective mechanisms that require induction. In addition, the decomposition products of peroxynitrite do not include hydrogen peroxide, the OxyR-requiring species that allows for gene induction of Prx. However, another member of the OxyR regulon is glutathione reductase (gor). This enzyme converts oxidized glutathione to its reduced form. Reduced glutathione can serve as an antioxidant for either ROS or RNS. Mutation of gor had no effect on peroxynitrite resistance (Fig. 6).

Co-infections are common among sexually transmitted diseases, especially co-infection by *N. gonorrhoeae* and *Chlamydia trachomatis* (Hillis *et al.*, 1994). *C. trachomatis* may benefit from the defensive mechanisms employed by the gonococcus to cope with oxidative and nitrosative stress, considering that RNS inhibit intracellular multiplication of *C. trachomatis* (Igietseme *et al.*, 1997). Studies have shown that local genital secretions from women infected with *N. gonorrhoeae* and concurrently with *Trichomonas vaginalis* and *C. trachomatis* have normal cytokine levels rather than the elevated levels normally associated with infection (Hedges *et al.*, 1998). This indicates that *N. gonorrhoeae* may be able to establish an immunosuppressive environment capable of protecting other infectious bacteria. The effect can be twofold, including the reduction of peroxynitrite and subsequent increase in bacterial viability (Fig. 8), as well as the ability of *N. gonorrhoeae* to reduce high levels of nitric oxide to an anti-inflammatory level, preventing NF-κB activation (Barth & Clark, 2008; Cardinale & Clark, 2005; Liaudet *et al.*, 2000).

In summary, the host immune system is capable of eliciting an assault on invading pathogens through the activity of a large collection of antimicrobial products. Some of these result in the generation of ROS and RNS, and the gonococcus shows high levels of resistance to both nitric oxide and peroxynitrite. Peroxynitrite resistance is a common mechanism within various gonococcal isolates and is not dependent on individual genes known to be involved in RNS defence in other bacterial species, suggesting a number of alternative possibilities. The fact that *N. gonorrhoeae* encodes such an expansive number of antioxidants means that creating mutants of each coding region is not a feasible technique to rapidly identify the mechanism of peroxynitrite resistance. This method would not be able to identify a novel mechanism. Furthermore, a change to a phenotype of peroxynitrite sensitivity may not be attainable without eliminating all of the resistance mechanisms. The hyperresistance of *N. gonorrhoeae* to peroxynitrite may be the result of a variety of systems. These may include biomolecules capable of scavenging peroxynitrite (i.e. glutathione), the enzymic reduction of the RNS (i.e. a novel reductase that has redundant activity with *ahpC*), repair of RNS-mediated damage (*msrA*, *recN*), or a combination of these mechanisms. Because high levels of peroxynitrite resistance do not occur in *N. meningitidis*, the gonococcal mechanism(s) responsible could be a function of variations in protein expression and/or genetic regulation between the two species or the result of a complete absence of a novel protective mechanism within the meningococcus. Current studies involve constructing a gonococcal library within *N. meningitidis*, where we will examine clones that give a significant increase in resistance to peroxynitrite.

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