Characterisation of the OxyR regulon of Neisseria gonorrhoeae

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ABSTRACT

OxyR regulates the expression of the majority of H₂O₂ responses in Gram-negative organisms. In a previous study we reported the OxyR dependent de-repression of catalase expression in the human pathogen *Neisseria gonorrhoeae*. In the present study we used microarray expression profiling of *N. gonorrhoeae* wild type strain 1291 and an oxyR mutant strain to define the OxyR regulon. In addition to katA (encoding catalase), only one other locus displayed a greater than two-fold difference in expression in the wild type:oxyR comparison. This locus encodes an operon of two genes, a putative peroxiredoxin/glutaredoxin (Prx) and a putative glutathione oxidoreductase (Gor). Mutant strains were constructed in which each of these genes was inactivated. A previous biochemical study in *N. meningitidis* had confirmed function of the glutaredoxin/peroxiredoxin. Assay of the wild type 1291 cell free extract confirmed Gor activity, which was lost in the gor mutant strain. Phenotypic analysis of the prx mutant strain in H₂O₂ killing assays revealed increased resistance, presumably due to up-regulation of alternative defence mechanisms. The oxyR, prx and gor mutant strains were deficient in biofilm formation, and the oxyR and prx strains had decreased survival in cervical epithelial cells, indicating a key role for the OxyR regulon in these processes.

INTRODUCTION

*N. gonorrhoeae*, the causative agent of the sexually transmitted infection gonorrhoea, is a host-adapted pathogen that poses a serious health threat worldwide. During infection, *N. gonorrhoeae* is exposed to oxidative stress in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by host defence mechanisms and as by-products of endogenous respiratory processes. These reactive species can damage all cellular macromolecules (*i.e.*, DNA, lipids and proteins) (reviewed by Imlay, 2003). *N. gonorrhoeae* is often associated with inflamed urogenital tissues and activated polymorphonuclear leukocytes (Archibald and Duong, 1986) which generate superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) as part of their bactericidal mechanism (reviewed by Burg and Pillinger, 2001; Hampton *et al.*, 1998). *N. gonorrhoeae* has evolved numerous defence mechanisms to sense and cope with this and other sources of oxidative stress that it encounters. Detoxification of H₂O₂ in *N. gonorrhoeae* depends on catalase (Tseng *et al.*, 2003), accumulation of manganese (Mn) by the MntABC transporter (Seib *et al.*, 2004) and cytochrome c peroxidase (Ccp) (Seib *et al.*, 2004; Turner *et al.*, 2003). Other defences involved in
protection against $\text{H}_2\text{O}_2$ include bacterioferritin (Bfr) (Chen and Morse, 1999) and methionine sulfoxide reductase (Msr) (Skaar et al., 2002; Taha et al., 1991; Wizemann et al., 1996).

$N.\text{gonorrhoeae}$ possesses very high constitutive levels of catalase (encoded by $\text{katA}$) (Hassett et al., 1990; Zheng et al., 1992) which are induced by $\text{H}_2\text{O}_2$ as a consequence of loss of OxyR repression (Tseng et al., 2003). An $\text{oxyR}$ mutant strain has nine-fold higher catalase activity than constitutive levels, four-fold higher activity than maximally induced wild type levels, and is significantly more resistant to $\text{H}_2\text{O}_2$ killing than the wild type (Tseng et al., 2003). This is distinct from the situation in $\text{Escherichia coli}$ and $\text{Salmonella typhimurium}$, in which OxyR is a positive regulator of catalase expression (Christman et al., 1985; Morgan et al., 1986; Pomposiello and Demple, 2001) and where increased sensitivity to $\text{H}_2\text{O}_2$ is seen in $\text{oxyR}$ mutant strains (Christman et al., 1985; Christman et al., 1989). OxyR of $N.\text{gonorrhoeae}$ contains all of the typical features of OxyR proteins; the LysR family helix-turn-helix motif, the active site cysteine residues and has 37%/59% sequence identity/similarity to OxyR of $\text{E. coli}$. In addition, $N.\text{gonorrhoeae}$ OxyR can complement an $\text{E. coli}$ $\text{oxyR}$ mutant strain and behave as an activator (Tseng et al., 2003).

OxyR belongs to the LysR family of DNA-binding transcriptional modulators (Christman et al., 1989) and has been extensively studied in $\text{E. coli}$ (reviewed by Pomposiello and Demple, 2001; Storz and Imlay, 1999). OxyR regulates expression of the majority of $\text{H}_2\text{O}_2$ responsive genes in $\text{E. coli}$, including $\text{katG}$ (hydroperoxidase I), $\text{ahpCF}$ (alkylhydroperoxide reductase), $\text{gorA}$ (glutathione reductase), $\text{grxA}$ (glutaredoxin 1), $\text{trxC}$ (thioredoxin 2), $\text{fur}$ (repressor of iron uptake), $\text{dps}$ (unspecific DNA binding protein), $\text{oxyS}$ (regulatory RNA), $\text{dsbG}$ (disulfide bond chaperone-isomerase) and $\text{fhuF}$ (protein required for iron uptake), $\text{hemH}$ (heme biosynthetic gene), six-gene $\text{suf}$ operon (may participate in Fe-S cluster assembly or repair), and $\text{uxuA}$ (mannonate hydrolase) (Pomposiello and Demple, 2001; Zheng et al., 2001a; Zheng et al., 2001b). These OxyR regulated genes have direct (e.g. removal of $\text{H}_2\text{O}_2$ by $\text{katG}$ and $\text{ahpC}$; control of redox balance by $\text{gor}$, $\text{grxA}$ and $\text{trxC}$) and indirect (e.g., control of the $\text{fur}$ and $\text{oxyS}$ regulators that affect numerous other genes) roles in defences against oxidative stress. The OxyR regulon of $\text{E. coli}$ was determined, in part, via DNA microarray-mediated transcription profiling of the $\text{H}_2\text{O}_2$ response (after exposure to 1 mM $\text{H}_2\text{O}_2$ for 10 min) of an $\text{E. coli}$ wild-type strain relative to an $\text{oxyR}$ mutant strain (Zheng et al., 2001b).

OxyR is constitutively expressed in $\text{E. coli}$ and $\text{S. typhimurium}$ (Storz et al., 1990; Zheng et al., 1998). $\text{H}_2\text{O}_2$ reversibly activates OxyR at the post-translational level through the oxidation of two cysteine residues and the formation of an intramolecular disulphide bond (Zheng et al., 1998). The disulfide bond is then reduced by glutaredoxin 1 (GrxA) and glutathione ($\gamma$-L-glutamyl-L-cysteinylglycine; GSH), which is in turn reduced by glutathione reductase (Gor), both of which are
part of the OxyR regulon in *E. coli* (Aslund and Beckwith, 1999). In this way oxyR expression is controlled via a negative feedback loop. OxyR-binding sites are unusually long (> 45bp) with limited sequence similarity. Both the oxidised and reduced forms of OxyR bind DNA, but OxyR uses two different modes of binding to enable it to act as both an activator and a repressor (Toledano *et al.*, 1994).

In this study we used a microarray approach to define the OxyR regulon of *N. gonorrhoeae* and enable further investigation of the peroxide stress response in this organism, including the role of the newly identified peroxiredoxin (Prx) and glutathione oxidoreductase (Gor).

**RESULTS**

**Characterisation of the OxyR regulon of *N. gonorrhoeae*: DNA microarray analysis and RT-PCR**

OxyR is known to regulate more than nine genes in *E. coli* that are involved directly or indirectly in the oxidative stress response (Zheng *et al.*, 2001b). To examine the OxyR regulon of *N. gonorrhoeae*, gene expression in a wild type *N. gonorrhoeae* strain 1291 and an isogenic 1291oxyR::kan mutant strain (Tseng *et al.*, 2003) was compared by analysis on *Neisseria gonorrhoeae/ Neisseria meningitidis* genome microarrays (TIGR). To rule out the possibility that suppressor mutations may have arisen in the key strain used in this study, 1291oxyR::kan, that may confuse interpretation of microarray analysis, we constructed a wild type oxyR “knock-in” version of the 1291oxyR::kan mutant strain (called “wild type*”) in which the wild type oxyR gene was used to replace the oxyR::kan allele (see Experimental procedures). The resulting 1291wild type* strains were compared to the parental 1291 strain and to 1291oxyR::kan to confirm that the 1291oxyR::kan H2O2 hyper-resistant phenotype (Tseng *et al.*, 2003) had returned to the parental 1291 wild type phenotype (result not shown). This confirmed that suppressor mutations were not responsible for the 1291oxyR::kan phenotype, but that the 1291oxyR::kan phenotype was solely due to inactivation of the oxyR gene.

Total RNA was isolated from wild type and 1291oxyR::kan mutant strain cultures that had been grown to exponential phase then exposed to 1 mM H2O2 for 10 min. Overall, three genes were differentially regulated by greater than two-fold (P value < 0.01) between the wild type and the oxyR mutant strain. Two genes, prx (NG0926, (LosAlamos, 2005)) and gor (NG0925), encoding a putative peroxiredoxin (Prx) and a putative glutathione oxidoreductase (Gor), respectively, were down-regulated in the oxyR mutant strain relative to wild-type (Table 1). The gene encoding catalase, katA (NG1767), was up-regulated in the oxyR mutant (Table 1) in accordance with
previous findings (Tseng et al., 2003). Results from the microarray analysis were confirmed using quantitative real time (RT)-PCR (Table 1).

The genes NG0926 and NG0925 have not previously been characterised in *N. gonorrhoeae* and are the focus of this study (see Figure 1A for a schematic of the genome region containing these genes). The predicted protein sequence of NG0926 is 98% identical to Prx of *N. meningitidis* which is able to reduce various peroxides, including H$_2$O$_2$, in the presence of GSH (Rouhier and Jacquot, 2003). NG0925 is annotated in the *N. gonorrhoeae* FA1090 genome (LosAlamos, 2005) as dihydrolipoamide dehydrogenase, the E3 component of the multienzyme pyruvate dehydrogenase complex (PDHC) which catalyses oxidative decarboxylation of α-ketoacids in the Krebs cycle. Protein family (Pfam) analysis (Bateman et al., 2002) places NG0925 in the pyridine nucleotide-disulphide oxidoreductase family (PF00070; E-value=2.6e-46) of which dihydrolipoyl dehydrogenase (EC 1.6.4.3) is a member. Other members of this family include glutathione reductase (Gor; EC 1.6.4.2), thioredoxin reductase (TR; EC 1.6.4.5) and mercuric reductase. These enzymes have high sequence and structural similarities and have a common mechanism, but have evolved different specificities. Due to the similarity of members of this family, we hypothesised that NG0925 encoded Gor, which has not previously been identified in *N. gonorrhoeae*, rather than the E3 component of the pyruvate complex.

The genes encoding the dihydrolipoyl dehydrogenase multienzyme complexes are usually organised in operons (de Kok et al., 1998); however NG0925 does not have genes encoding the E1 and E2 components adjacent to it. Several dihydrolipoyl dehydrogenases have already been described or identified in the pathogenic Neisseria. The PDHC of *N. meningitidis* is encoded by NMB1341 (*pdhA*, E1 component), NMB1342 (*aceF*, E2 component) and NMB1344 (*lpdA*, E3 component) (Ala' Aldeen et al., 1996; Tettelin et al., 2000), which correspond to NG0565, NG0564 and NG0562 in *N. gonorrhoeae* (LosAlamos, 2005). The 2-oxoglutarate dehydrogenase complex (OGDHC) of *N. gonorrhoeae* has been annotated in the genome and is encoded by NG0915 (*ddH*, E3 component), NG0916 (*sucB*, E2 component) and NG0917 (*sucA*, E1 component). The succinate dehydrogenase (SDH) is located downstream and is encoded by NG0920 (*dhsB*, iron-sulfur protein), NG0921 (*dhsA*, flavoprotein subunit), NG0922 (*dhsD*, hydrophobic membrane anchor) and NG0923 (*dhsC*, cytochrome b556 chain). The E3 component can be shared by different complexes, however the E3 gene NG0562 is unique for PDHC, and OGDHC contains the usual E3 gene NG0915 (de Kok et al., 1998). Also, disruption of the E3 gene of PDHC in *N. meningitidis* results in loss of PDHC, but not OGDHC, activity (de Kok et al., 1998). This in silico analysis suggests that NG0925 does not encode the E3 component of the pyruvate dehydrogenase. Further analysis of a gor mutant strain supports the suggestion that it encodes Gor and is described below.
Hydrogen peroxide-dependence of expression of the OxyR regulon

Studies in *E. coli* have shown that H$_2$O$_2$ reversibly activates OxyR at the posttranslational level through the oxidation of two cysteine residues and the formation of an intramolecular disulfide bond (Zheng *et al.*, 1998). To investigate the H$_2$O$_2$-dependence of OxyR in *N. gonorrhoeae*, expression of the genes within the OxyR regulon was investigated by RT-PCR in the wild type strain under growth conditions ± 1 mM H$_2$O$_2$. H$_2$O$_2$ induced expression of *prx* (6.4-fold), *gor* (8-fold) and *katA* (4.65-fold) (Table 1). These data, in conjunction with the *oxyR* mutant:wild type expression ratios from microarray analysis, indicate that *gor* and *prx* are activated by OxyR under conditions of increased H$_2$O$_2$. On the other hand, OxyR derepresses catalase expression upon exposure to increased levels of H$_2$O$_2$. This supports earlier reports based on catalase activity assays in the *N. gonorrhoeae oxyR* mutant strain (Tseng *et al.*, 2003).

Analysis of *prx* and *gor* transcription

Co-transcription of *prx* and *gor* was investigated due to their close proximity, their potentially related roles within the cell and the similarity of their transcription profile by DNA microarray and RT-PCR analysis (Table 1). To confirm that the two genes are in fact part of an operon, total RNA from *N. gonorrhoeae* wild-type strain was used in reverse transcription PCR experiments. Three pairs of primers were used: two pairs were designed to amplify the individual *prx* and *gor* genes, and the third pair spans the intergenic region to demonstrate co-transcription of the *prx* and *gor* genes. All three RT-PCR products were the expected size for cotranscription (see Figure 1D). There was no amplification when reverse transcriptase was omitted from the reaction, indicating that the PCR products seen were not a result of contamination of the RNA sample with genomic DNA. Further investigation of *prx* and *gor* transcription indicated that they are not co-transcribed with their flanking genes, *metE* and *dhsC* respectively (data not shown; see Figure 1A for map of *prx* and *gor* region in the genome). *dhsC* is part of the SDH complex. The fact that *gor* and *dhsC* are not co-transcribed provides further support that *gor* does not encode the dihydrolipoamide dehydrogenase component of the PDHC, as is annotated in the genome database (see above for a full description). Further RT-PCR experiments were conducted comparing the *prx-gor* operon expression in 1291 wild type, 1291 wild type* and 1291oxyR::kan (see Figure 1D). The *prx* transcript, reduced in the 1291oxyR::kan mutant returned to 1291 wild type levels in the 1291
wild type* strain, further confirming that the \textit{1291oxyR::kan} regulatory phenotype was not due to second site suppressors mutations.

**Construction of \textit{N. gonorrhoeae} prx and gor mutant strains**

To investigate the role of Prx and Gor in \textit{N. gonorrhoeae}, prx and gor mutant strains were constructed via insertion of a kanamycin resistance cassette into the ORF of the \textit{prx} and \textit{gor} genes of \textit{N. gonorrhoeae} strain 1291 (Figure 1C). These mutant strains were confirmed by PCR analysis with the primers preprx and prx\_R for the \textit{prx} mutant strain or preprx and gor\_R for the \textit{gor} mutant strain (Table 2). The growth characteristics of the \textit{N. gonorrhoeae} wild type and the \textit{prx}, \textit{gor} and \textit{oxyR} mutant strains were indistinguishable under aerobic conditions in Brain Heart Infusion broth (BHI; Oxoid) at 37°C as monitored by the increase in optical density at 600 nm. Growth studies were conducted in triplicate and repeated on two occasions (data not shown).

**Glutathione reductase activity is absent in the gor mutant strain**

To determine the physiological function of NG0925, Gor activity was measured in cell free extracts of overnight cultures of \textit{N. gonorrhoeae} wild type, \textit{gor} and \textit{prx} mutant strains. Gor catalyses the reaction \(2\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+\). The reduction of GSSG, and thus Gor activity, can be measured indirectly by following the consumption of NADPH, measured as a decrease in absorbance at 340 nm. This enzyme assay showed Gor activity in the wild type strain, which increased with increasing protein concentration (Figure 2) and increasing GSSG concentration (data not shown). No significant Gor activity was present in the \textit{gor} mutant strain relative to the wild type strain (see Figure ). These results indicate that NG0925 does encode the Gor of \textit{N. gonorrhoeae}. Gor activity in the \textit{prx} mutant strain was identical to the wild type levels (data not shown; P value =1). These results indicate that Prx is not required for Gor activity and that the phenotype of the \textit{gor} mutant strain is not a result of a polar effect from the \textit{prx} mutation.

The apparent \(K_m\) \((K_{\text{m,app}})\) for the reduction of GSSG by \textit{N. gonorrhoeae} cell free extracts was calculated using Lineweaver-Burke (454±120 µM) and Eadie-Hofstee plots (475±132 µM).

**Role of Prx and GOR in defence against oxidative stress**

The \textit{oxyR} mutant of \textit{N. gonorrhoeae} is highly resistant to \text{H}_2\text{O}_2 stress (Tseng \textit{et al.}, 2003). This resistance is presumably in part due to the increased catalase activity seen in the \textit{oxyR} mutant
strain (Tseng et al., 2003); a katA mutant of *N. gonorrhoeae* is highly sensitive to \( \text{H}_2\text{O}_2 \) (Seib et al., 2004). To investigate the role of the two other OxyR-regulated proteins in the oxidative stress response, \( \text{H}_2\text{O}_2 \), xanthine-xanthine oxidase, paraquat and cumene hydroperoxide killing assays were performed using *N. gonorrhoeae* wild type and prx and gor mutant strains. The prx mutant strain was significantly more resistant to killing with \( \text{H}_2\text{O}_2 \) than the wild-type strain (\( P \leq 0.05 \); Figure 3), while the gor mutant strain was only slightly more resistant than the wild type. Both mutant strains behaved like the wild type strain in the xanthine/xanthine oxidase, paraquat and cumene hydroperoxide assays (data not shown). RT-PCR analysis of catalase transcript levels showed a 2.3±0.2 increase in expression of katA in the prx mutant strain relative to the wild type strain, which could account for the increased \( \text{H}_2\text{O}_2 \) resistance seen in this strain.

**oxyR and gor mutant strains have decreased survival in cervical epithelial cells**

To determine the ability of *N. gonorrhoeae* wild type, oxyR, prx, gor and katA mutant strains to associate with, invade and survive within primary human ectocervical epithelial (pex) cells, they were challenged with either the wild type or mutant strains and infection allowed to progress for 2 h (37 °C, 5% CO\(_2\)). There was a small but significant difference observed in the ability of the oxyR and gor mutant strains to associate with pex cells upon comparison to wild type gonococci. The oxyR and gor mutants showed a more significant decrease in invasion and survival over two hours, relative to wild type (Figure 4). However, there was no statistically significant difference in the mean percent association or survival of the katA and prx mutant strains relative to *N. gonorrhoeae* strain 1291 wild type (Figure 4).

**oxyR, gor and prx mutant strains have decreased biofilm formation**

Studies performed in continuous-flow chambers have recently shown that *N. gonorrhoeae* strain 1291 can form a biofilm on glass coverslips as well as on primary cervical cells without loss of viability of the epithelial cells (Greiner et al., 2005). The ability of the *N. gonorrhoeae* 1291 oxyR, prx and gor mutant strains to form a biofilm was investigated via confocal microscopy after two days of growth in continuous flow chambers. All three mutant strains had a significant decrease in biofilm formation relative to the wild type strain; oxyR, prx and gor formed approximately 7%, 3% and 9% of the wild type biofilm biomass, respectively (Figure 5).
DISCUSSION

*N. gonorrhoeae* encounters significant levels of ROS, including H$_2$O$_2$, within the female urogenital tract as a result of exposure to resident lactic acid bacteria (Whittenbury, 1964) and activated polymorphonuclear leukocytes (PMNs) (Archibald and Duong, 1986). The peroxide stress response of *N. gonorrhoeae* is unusual in that it contains two H$_2$O$_2$ responsive regulators of oxidative stress defences, OxyR (Tseng et al., 2003) and PerR (Wu et al., 2006). OxyR is typically found in Gram-negative bacteria such as *E. coli* and *S. typhimurium* (Christman et al., 1989), while PerR typically regulates peroxide stress responses in Gram-positive organisms including *Bacillus subtilis* (Bsat et al., 1998) and *Staphylococcus aureus* (Horsburgh et al., 2001). The recently defined PerR regulon in *N. gonorrhoeae* includes 12 genes, several of which have a proven or suggested role in defence against ROS (Wu et al., 2006). Here we define the relatively small OxyR regulon of *N. gonorrhoeae* and describe two previously uncharacterised proteins of *N. gonorrhoeae*, Prx and Gor. All three OxyR regulated genes are upregulated in response to H$_2$O$_2$ stress, indicating that they play a role in protecting *N. gonorrhoeae* from damage caused by H$_2$O$_2$.

OxyR regulates more than ten genes in *E. coli*, including katG (hydroperoxidase I) ahpCF (alkylhydroperoxide reductase), and gorA (glutathione reductase). It has been suggested that the Prx system functionally replaces the well-known *E. coli* AhpCF system (Vergauwen et al., 2003), which is the primary scavenger of endogenous H$_2$O$_2$ in *E. coli*. (Seaver and Imlay, 2001). Although the OxyR regulon of *N. gonorrhoeae* contains genes that are regulated by OxyR in *E. coli*, the *N. gonorrhoeae* regulon is much smaller and peroxide stress response of *N. gonorrhoeae* appears to be quite distinct from that of *E. coli*. For instance, an oxyR mutant strain of *N. gonorrhoeae* is highly resistant to H$_2$O$_2$ stress (Tseng et al., 2003), while sensitivity to H$_2$O$_2$ is seen in an oxyR mutant of *E. coli* (Christman et al., 1985; Christman et al., 1989). OxyR mutant strains of *Pseudomonas aeruginosa* (Ochsner et al., 2000), *Haemophilus influenzae* (Maciver and Hansen, 1996), *Xanthomonas campestris* (Mongkolsuk et al., 1998) and *Brucella abortus* (Kim and Mayfield, 2000) are also hypersensitive to oxidative stress.

Prx are non-heme peroxidases that catalyse the reduction of alkyl hydroperoxides via reactive cysteines. The cysteines are then regenerated via thioredoxin (Trx) or Grx, which in turn are reduced by NADPH and thioredoxin reductase (TR) or NADPH, GSH and Gor (Poole, 2005). GSH is considered one of the first lines of defence against oxidative stress (Pomposiello and Demple, 2002). The reduced pool of GSH within the cell is typically maintained by Gor using NADPH as reductant (Carmel-Harel and Storz, 2000). NADPH is then recycled by glucose-6-phosphate dehydrogenase (Hofmann et al., 2002). Therefore, the finding that prx and gor are transcriptionally linked and coordinately regulated in *N. gonorrhoeae* is appropriate in light of their
coordinated function. The Prx protein identified in *N. gonorrhoeae* is 98% identical to the hybrid Prx (N-terminus Prx domain and C-terminus Grx domain) characterised in *N. meningitidis* which reduces various peroxides, including H$_2$O$_2$, in the presence of GSH (Rouhier and Jacquot, 2003). Both domains possess biological activity; the reducing power of GSH regenerates the catalytic cysteine of Prx via the Grx domain (Rouhier and Jacquot, 2003). The location of these domains in the *N. gonorrhoeae prx* ORF is shown in Figure 1. The hybrid Prx has been identified in several other bacteria including *H. influenzae* (Vergauwen et al., 2003), *Vibrio cholerae* (Cha et al., 2004) and *Chromatium gracile* (Vergauwen et al., 2001).

Prxs are divided into three classes: typical and atypical 2-Cys Prxs, and 1-Cys Prxs. These classes share the same initial catalytic mechanism; an active site cysteine (the peroxidatic cysteine) is oxidized to a sulfinic acid by the peroxide substrate. The mechanism by which the thiol is regenerated from the sulfinic acid back is what distinguishes the three enzyme classes (Wood et al., 2003). Phylogenic analysis indicated that Prx of *N. meningitidis* is grouped with the Prx of *V. cholera* in the atypical 2-Cys class of Prx (Cha et al., 2004). In the atypical 2-Cys Prxs, both the peroxidatic cysteine and its corresponding resolving cysteine are contained within the same polypeptide, and catalysis involves the formation of an intramolecular disulfide bond (Cha et al., 2004). Due to the homology between the *N. gonorrhoeae* and *N. meningitidis* Prx, it follows that a similar catalytic mechanism would be used by the *N. gonorrhoeae* Prx.

The hybrid Prx proteins are all capable of reducing H$_2$O$_2$, tert-butylhydroperoxide and cumene hydroperoxide (Cha et al., 2004; Pauwels et al., 2004; Vergauwen et al., 2001; Vergauwen et al., 2003). In addition, the *H. influenzae* Prx is able to protect supercoiled DNA against the metal ion-catalysed oxidation-system (Pauwels et al., 2003). The *prx* mutant strain of *N. gonorrhoeae* had increased resistance to H$_2$O$_2$ relative to the wild type strain. A similar result was seen in *H. influenzae*, and was attributed to the presence of elevated levels catalase (HktE) in the absence of a functional *pgdx* gene (Pauwels et al., 2004). Catalase transcript levels were also upregulated 2.3 fold in the *N. gonorrhoeae prx* strain relative to the wild type strain. These findings indicate that the absence of Prx, which is believed to fulfil a role as a major peroxidase for low concentrations of H$_2$O$_2$ (Pauwels et al., 2004), result in increased H$_2$O$_2$ levels that causes derepression of catalase in *N. gonorrhoeae*.

Gor (NAD(P)H:oxidised-glutathione oxidoreductase) is nearly ubiquitous and has been well characterised in many organisms including *E. coli* and *Saccharomyces cerevisiae* (Carmel-Harel and Storz, 2000). Gor plays a central role in maintaining the redox balance of the cell. Gor typically maintains the reduced pool of GSH (Carmel-Harel and Storz, 2000), which is a low molecular weight compound (γ-L-glutamyl-L-cysteinylglycine) that is considered one of the first lines of
defence against oxidative stress (Pomposiello and Demple, 2002). GSH, typically present in cells in millimolar concentrations (5mM in E. coli) (Prinz et al., 1997), is a chemical scavenger of radicals and acts as a hydrogen donor to restore oxidized macromolecules (Carmel-Harel and Storz, 2000). Very high concentrations of GSH (17.3 mM) are present in N. gonorrhoeae, which may constitute a powerful antioxidant system (Archibald and Duong, 1986). Despite the proposed importance of GSH as an antioxidant in N. gonorrhoeae, Gor has not been identified in this organism until now. The apparent K_m determined for Gor (454±120 µM) is consistent with the high intracellular concentrations of GSH. The gor mutant strain constructed in this study had no significant levels of Gor activity, and showed a slight increase in resistance to H_2O_2 killing relative to the wild type strain. In E. coli, gor mutants also had increased resistance to paraquat and H_2O_2 (Becker-Hapak and Eisenstark, 1995; Kunert et al., 1990). Unlike the situation in a prx mutant (Pauwels et al., 2004), catalase levels were the same in the E. coli gor mutant strain as the wild type, but it was proposed that the increased resistance to H_2O_2 may have been a result of upregulation of GSH biosynthetic genes (Becker-Hapak and Eisenstark, 1995).

H_2O_2-dependent regulation of gor expression was also observed in a study of the transcriptional response of N. gonorrhoeae to H_2O_2 that was published during the preparation of this manuscript (Stohl et al., 2005). This study found that the expression of 75 genes was upregulated after transient exposure to H_2O_2, including gor (annotated as dldH), katA and several other genes involved in oxidative stress defence, the heat shock response, iron uptake, DNA repair and energy metabolism (Stohl et al., 2005).

The H_2O_2 resistance of the N. gonorrhoeae oxyR mutant strain (Tseng et al., 2003) is presumably largely due to the increased catalase expression seen in the oxyR mutant strain since a katA mutant of N. gonorrhoeae is highly sensitive to H_2O_2 (Seib et al., 2004). A similar situation may also explain the increased resistance of the prx mutant strain to H_2O_2 killing. The complex nature and the fine balance of the oxidative stress response is indicated by similar but reversed findings in E. coli: increased sensitivity to H_2O_2 is seen in a strain overexpressing ahpCF on a plasmid (Storz et al., 1989). It is proposed that the increased ahpCF expression may cause the OxyR regulator to be titrated away from other OxyR-regulated genes, including katG, which confer resistance to high levels of exogenous H_2O_2 (Storz et al., 1989). The importance of this balance in vivo is implied by the finding from the ex vivo assays of pex cell survival and biofilm formation. Oxidative killing mechanisms have not yet been fully explored in cervical epithelial cells, but intestinal and airway epithelial cells are known to be able to kill bacteria by oxidative mechanisms (Battistoni et al., 2000; Rochelle et al., 1998; Schmidt and Walter, 1994). Despite the increased resistance of the oxyR mutant to in vitro oxidative killing, assumed to be due to increased expression of catalase, this strain had decreased survival in pex cells even though the katA mutant
strain showed no decrease in survival compared to wild type. The gor, but not prx, mutant strain replicates the oxyR phenotype indicating that Gor and GSH play an important role in survival of *N. gonorrhoeae* within pex cells.

The *N. gonorrhoeae* oxyR, prx and gor mutant strains have decreased ability to form a biofilm. It has been suggested that the formation of a biofilm by *N. gonorrhoeae* may contribute to its ability to persist in an asymptomatic state in the female genital tract (Hook and Handsfield, 1999). Indeed, the number of human infections known to involve bacterial biofilms is increasing, as is the understanding of the metabolic alterations which occur during biofilm growth (reviewed in Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004). Bacteria within biofilms display increased resistance to antimicrobial agents, and links between biofilm formation and oxidative stress defences have been seen in several microbes including *E. coli* (Schembri *et al.*, 2003), *H. influenzae* (Murphy *et al.*, 2005), *P. aeruginosa* (Sauer *et al.*, 2002), *Campylobacter jejuni* (Sampathkumar *et al.*, 2006), *Streptococcus mutans* (Wen *et al.*, 2005), *Burkholderia pseudomallei* (Loprasert *et al.*, 2002) and *Candida albicans* (Murillo *et al.*, 2005). Of particular interest, the Prx of *H. influenzae* (73% similarity/81% identity to the *N. gonorrhoeae* Prx over the entire predicted amino acid sequence) is expressed in greater abundance during biofilm growth and Prx deficient mutant strains have 25–50% reduction in biofilm formation compared to the parent strains (Murphy *et al.*, 2005). In yeast, the gene encoding glutamylcysteine synthase, an important gene in GSH synthesis, is upregulated during early stages of biofilm development (Murillo *et al.*, 2005). In *E. coli*, the OxyR-regulated adhesin Ag43 promotes biofilm formation (Danese *et al.*, 2000; Kjaergaard *et al.*, 2000). Ag43 is repressed by OxyR, however expression is derepressed upon exposure to oxidative stress (Schembri *et al.*, 2003). *E. coli agn43* mutant strains are defective in biofilm formation in glucose-minimal medium compared to wild-type strains, whereas oxyR mutant strains have increased Ag43 expression and increased biofilm formation (Danese *et al.*, 2000; Schembri *et al.*, 2003). Ag43-mutant cells were sensitive to H$_2$O$_2$; Ag43 mediated cell aggregation is believed to confer protection against H$_2$O$_2$ killing (Schembri *et al.*, 2003). A similar situation is seen in *B. pseudomallei*, where oxyR mutant strains are hypersensitive to H$_2$O$_2$ and paraquat and have increased biofilm formation in minimal medium (Loprasert *et al.*, 2002). Oxidative stress defences are also induced during immobilised or biofilm growth in *C. jejuni* (Sampathkumar *et al.*, 2006) and *P. aeruginosa* (Sauer *et al.*, 2002).

While the underlying mechanisms linking oxidative stress defences and biofilm formation is not yet known, it has been argued that complex interactions between pathogens and the host inflammatory response results in modification of the host environment which induce biofilm formation (Hall-Stoodley *et al.*, 2004). Oxidative stress in the host may be a trigger for the upregulation of oxidative stress defences and biofilm formation as a complex and linked defence
strategy. These findings, in conjunction with the pex cell results described above, provide interesting insights into the in vivo survival mechanisms of N. gonorrhoeae.

EXPERIMENTAL PROCEDURES

Strains and growth conditions.

N. gonorrhoeae strain 1291 and the oxyR mutant derivative, N. gonorrhoeae oxyR::kan (Tseng et al., 2003), were used in this study. N. gonorrhoeae strain 1291 is an American type culture collection (ATCC) strain that was isolated from a male patient with gonococcal urethritis. N. gonorrhoeae was grown on Brain Heart Infusion agar or broth (BHI; Acumedia) supplemented with 10% Levinthal’s base and 1% IsoVitaleX (Becton Dickinson) at 37 °C in 5% CO2. Escherichia coli strain DH5α was cultured in LB broth or on LB plates containing 1.5% bacteriological agar (Difco). Ampicillin and kanamycin were used at a final concentration of 100 µg ml⁻¹.

Recombinant DNA techniques and nucleotide sequence analysis

Recombinant DNA techniques were used as described in Sambrook et al. (1989). PCR was essentially done as described by Saiki et al. (1988). Primers used were as described by Tseng et al. (Tseng et al., 2003) or as listed in (Table 2). Nucleotide sequence analysis was performed using MacVector (Oxford Molecular). DNA and protein alignments were performed using ClustalW (Jeanmougin et al., 1998). All restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (NEB).

Construction of knockout mutants of the prx and gor genes of N. gonorrhoeae

Knockout constructs of the prx and gor genes were made via insertion of a kanamycin-resistant cassette (pUC4Kan; Amersham Biosciences) into a suitable unique restriction site in the coding region of each gene. The prx and gor genes were amplified from N. gonorrhoeae strain 1291 using primers prxA and gorB (Table 2) and cloned into pGEM®-T Easy (Promega), generating pGEM-Prx/Gor. The prx and gor insertional mutations were created by digesting pGEM-Prx/Gor with HpaI and EcoRV respectively and ligating the isolated HincII kanamycin-cassette-containing fragment derived from pUC4Kan (see Figure 1). These constructs were linearised with NotI and N. gonorrhoeae strain 1291 was transformed with each of the prx::kan and gor::kan knockouts as described previously (Jennings et al., 1995). Multiple, independent mutant strains were isolated.
and confirmed by PCR using the primers preprx and kan_do (Table 2). Previous work has demonstrated that the pUC4kan kanamycin cassette has no promoter or terminator that is active in Neisseria and will neither affect transcription nor have a polar effect on expression of adjacent genes (Jennings et al., 1995; van der Ley et al., 1997). To rule out the possibility that suppressor mutations may have arisen in 1291oxyR::kan, we constructed a wild type oxyR “knock-in” version “wild type*” of the 1291oxyR::kan mutant strain. Knock-in strains, in which the wild type oxyR gene was used to replace the oxyR::kan allele, were made by transforming NotI linearised pHJToxyR (Tseng et al., 2003), which contains the wild type oxyR gene, into the 1291oxyR::kan mutant strain, and then selecting for a kanamycin sensitive phenotype by replica plating. Confirmation that the oxyR::kan allele had been replaced by wild type oxyR via homologous recombination was confirmed by amplification and sequencing of the oxyR gene. The resulting 1291wild type* strains were analysed by comparison to the parental wild type 1291 strain and to 1291oxyR::kan, to confirm that both the 1291oxyR::kan H₂O₂ resistant phenotype (Tseng et al., 2003), and regulatory phenotypes seen by non-quantitative PCR (regulation of prx; see Results section) had returned to the 1291 wild type phenotype, thus confirming that suppressor mutations were not responsible for the 1291oxyR::kan phenotype, but that the 1291oxyR::kan phenotype was solely due to inactivation of the oxyR gene.

Microarray analysis.

Triplicate cultures of N. gonorrhoeae strain 1291 wild type and the oxyR mutant were grown to exponential phase (optical density at 600 nm = 0.2 to 0.5). These cultures were then exposed to 1mM hydrogen peroxide for 10 min prior to RNA extraction. Approximately 100 µg of total RNA was prepared from each sample using the RNeasy Maxi Kit according to the manufacturer's instructions (Qiagen). The triplicate samples were pooled and the integrity and concentration of RNA was determined via micro-fluidic analysis on a bio-analyser (Agilent Technologies).

All microarray analysis was performed on N. gonorrhoeae/meningitidis genome arrays (TIGR; http://pfgrc.tigr.org/). Each microarray consists of 6,389 70mer oligonucleotides representing open reading frames (ORFs) from N. gonorrhoeae strains FA1090 and ATCC 700825 (reference strain), and N. meningitidis strains Z2491 (serogroup A) and MC58 (serogroup B). N. gonorrhoeae strain 1291 was used in this study to enable comparison with previous reports on OxyR and oxidative stress responses of N. gonorrhoeae from this laboratory.

5 µg of each total RNA sample was labelled using random hexamers and direct incorporation of fluorescently Cy3- or Cy5-labelled nucleotides as previously described (Grimmond
The hybridisations were performed in triplicate and incorporated a dye-swap to account for dye bias. After 16 hrs of hybridisation, the arrays were washed and scanned on an Agilent G2565BA microarray scanner at a 5 micron resolution. The resulting images of the hybridisations were analysed using Imagene 5.5 (BioDiscovery Inc.) and the mean foreground, mean background and spot/signal quality determined.

All primary data was imported into an in-house installation of the comprehensive microarray relational database, BASE (http://kidney.scgap.org/base) (login: oxyR, password: oxyR). After print-tip intensity independent Lowess normalisation, differential expression was defined using a robust statistical method rather than simple fold change. All genes were ranked using the B statistic method where both fold change and variance of signals in replicates is used to determine the likelihood that genes are truly differentially expressed. A threshold in the B statistic of 0.0 was adopted as genes with a B score>0 have a >50% probability of being truly differentially expressed (Smyth et al., 2003). The ranked B-scores for all genes in each experiment are also maintained in BASE.

Quantitative Real-time PCR.

Total RNA was isolated by using the RNeasy kit (Qiagen) as described above. Cultures were exposed to 1mM hydrogen peroxide for 10 min prior to RNA extraction. The equivalent of 1 µg of the total RNA preparation was treated with RQ1 RNase-free DNase (Promega). RNA was reverse transcribed using random primers and the TaqMan® RT-PCR kit (PE Applied Biosystems) as recommended by the manufacturer. Primers were designed using Primer Express 1.0 software (ABI Prism; PE Biosystems). All Real-Time PCR reactions were performed in triplicate in a 25 µl mixture containing cDNA (5µl of 1/5 dilution), 1X SYBR Green buffer (PE Applied Biosystems) and approximately 2 µM of each primer (see Table 2 for primer sequences). 16S rRNA was used as the standard control in each quantitative PCR. Amplification and detection of specific products were performed with the ABI Prism 7700 sequence detection system (PE Applied Biosystems) with the following cycle profile: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Data was analysed with ABI Prism 7700 v1.7 analysis software. Relative gene expression between the N. gonorrhoeae wild-type strain and the N. gonorrhoeae oxyR mutant strain was determined using the 2^\Delta\DeltaCT relative quantification method. To investigate H2O2-dependence of OxyR in N. gonorrhoeae, expression of the genes within the OxyR regulon was also investigated in the wild type strain under growth conditions ±1 mM H2O2.

et al., 2000).
Semi-quantitative Real-time PCR

PCR was carried out in 50µl reactions using 1X Taq buffer, 1.5 mM MgCl₂, 1 unit of Taq DNA polymerase (Promega), cDNA (prepared as described above) and gene specific primers designed for quantitative RT-PCR (Table 2) with the following cycling conditions: 30 cycles of 94°C for 30 s, 50°C for 30s, 72°C for 30 s and 1 cycle of 72°C for 10 min. 16S rRNA was used as an internal standard. PCR products were run on a 2% agarose gel.

Glutathione reductase assay

Cell free extracts of overnight cultures of N. gonorrhoeae strain 1291 and the gor mutant were prepared by resuspending cells in phosphate buffered saline (PBS), followed by three cycles of freezing and thawing. Unbroken cells were removed by centrifugation and the supernatant was collected. Protein concentration in cell extracts was determined by absorbance at 280nm. The assay mixture included 50mM K₂HPO₄/0.1mM EDTA pH 7.5, 100µM NADPH, 1mM GSSG and cell free extract in 1 ml. The decrease in [NADPH] was followed at 340 nm using 6220 as the molar extinction coefficient.

Oxidative stress killing assays

Paraquat, xanthine/xanthine oxidase and H₂O₂ (Johnson et al., 1993) killing assays were performed using established methods as described by Tseng et al. (Tseng et al., 2001). Briefly, cells from agar plates were harvested, resuspended in PBS and 10⁵ to 10⁷ cells were added to a solution of BHI broth to a final volume of 100µl. The killing assay was started by the addition of a final concentration either 10mM paraquat (Sigma), or 4.3 mM xanthine and 300 mU/ml xanthine oxidase (Sigma), or 10 mM H₂O₂ (Riedel-de Haen). Cultures were incubated at 37°C/5% CO₂ and at various time points samples were taken, plated onto BHI agar after serial dilutions and incubated at 37 °C in 5% CO₂. Experiments were done in triplicate and repeated on several occasions. Cumene hydroperoxide killing assays were also performed as described above using 0.005-0.1 % cumene hydroperoxide.

Primary human ectocervical epithelial (pex) cell survival assay.

Primary human ectocervical epithelial (pex) cells were procured and maintained as described previously (Edwards et al., 2000) and cell monolayers were grown to confluence in 35 mm tissue culture dishes (Falcon). To determine the ability of N. gonorrhoeae wild type and oxyR mutant strains to associate with, invade and survive within pex cells, they were challenged with either the wild type or mutant strain and infection allowed to progress at 37 °C, 5% CO₂. For
association assays the infection medium was removed, and the cells rinsed with PBS. For invasion assays, pex cells were incubated for a further 30 min with medium containing 100 µg of gentamicin (Gibco) per ml to kill extracellular bacteria. Survival assays were performed in a similar manner with the exception that following gentamicin treatment the infected cell monolayers were again rinsed with PBS. Fresh antibiotic-free medium was then added to each infected cell monolayer before 1h or 2h incubation. Following each assay, pex cells were lysed with 0.5% saponin to release invasive bacteria, and serial dilutions were plated to determine CFUs. The percent invasion was determined as a function of the original inoculum. P-values were determined using a Kruskal-Wallis non-parametric analysis of variance.

**Biofilm formation by *N. gonorrhoeae***

For examination of biofilm formation via confocal microscopy, the *N. gonorrhoeae* 1291 wild type and the gor, prx, and oxyR mutant strains were transformed with a plasmid encoding a green fluorescent protein (GFP; pLES98 containing GFP was a gift from Virginia Clark at the University of Rochester, NY). Strains were propagated from frozen stock cultures on GC agar with 10 ml/L IsoVitaleX (Becton-Dickinson, Franklin Lakes, NJ), and incubated at 37°C and 5% CO₂. Overnight plate cultures were used to create cell suspensions for inoculation of biofilm flow chambers.

*N. gonorrhoeae* was grown in continuous flow chambers in 1:10 GC broth (Kellogg *et al*., 1963) diluted in PBS with 1% IsoVitaleX, 100 µM sodium nitrite, and 5 µg/µl chloramphenicol to maintain pGFP. Cell suspensions of 2x10⁸ CFU/ml (in approximately 1 ml of biofilm media) were used to inoculate 37x5x5 mm flow cell chamber wells. These chambers were designed to reduce fluid shear on biofilm (versus typical 1 mm depth wells). Flow chambers were incubated under static conditions at 37°C for 1 hour post-inoculation. Chambers were then incubated for another 48 hours under 180 µl/min flow. After 48 hours, the biofilm effluent was cultured to assure culture purity, and biofilm formation was assessed via confocal microscopy.

Z-series photomicrographs of flow chamber biofilms were taken with the Nikon PCM-2000 confocal microscope scanning system (Nikon Inc., Melville, NY) using a modified stage for flow cell microscopy. GFP was excited at 450-490 nm for biofilm imaging. Three dimensional volume images were rendered using Nikon’s accompanying EZ-C1 software. Each z-series photomicrograph was saved as a series of tiff images that were converted into 8-bit grayscale images using ImageJ software (Abramoff *et al*., 2004) (available free from the NIH through http://rsb.info.nih.gov/ij), for analysis in COMSTAT (Heydorn *et al*., 2000) (http://www.cbm.biocentrum.dtu.dk/English/Services/Resources/COMSTAT.aspx). An info file
(including pixel sizes for the x, y, z axes, and the number of the starting image and the total number of images in the stack) was created for each series of tiff images to direct COMSTAT to which images to analyse. COMSTAT was then used to threshold the images to reduce background. Biomass, and average and maximum thickness in each z-series was calculated by COMSTAT from the threshold images.

ACKNOWLEDGEMENTS

This work was supported by Program Grant 284214 from the National Health and Medical Research Council of Australia. SMG is a recipient of a NHMRC Career Development award and a senior research affiliate of the ARC Special Research Centre for Functional and Applied Genomics. The authors would like to thank NIH and TIGR for the provision of the Neisseria arrays. We acknowledge the Gonococcal Genome Sequencing Project supported by USPHS/NIH grant #AI38399, and B.A. Roe, L. Song, S. P. Lin, X. Yuan, S. Clifton, Tom Ducey, Lisa Lewis and D.W. Dyer at the University of Oklahoma. The GenBank accession number for the completed N. gonorrhoeae strain FA1090 genome is AE004969.

REFERENCES


Table 1. Differentially expressed genes in *N. gonorrhoeae* wild type versus the *oxyR* mutant.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>Microarray</th>
<th>Microarray Validation</th>
<th>H(_2)O(_2) regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>oxyR : WT</td>
<td>B-Stat</td>
<td>oxyR : WT</td>
</tr>
<tr>
<td><strong>Reduced expression in <em>oxyR</em> mutant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NG0925</td>
<td>Glutathione reductase (<em>gor</em>)</td>
<td>0.32</td>
<td>6.24</td>
<td>0.11±0.015</td>
</tr>
<tr>
<td>NT01NG1498</td>
<td>ORF (5' end of <em>gor</em>)</td>
<td>0.35</td>
<td>6.38</td>
<td>ND</td>
</tr>
<tr>
<td>NG0926</td>
<td>Peroxiredoxin (<em>prx</em>)</td>
<td>0.31</td>
<td>6.10</td>
<td>0.11±0.004</td>
</tr>
<tr>
<td><strong>Increased expression in <em>oxyR</em> mutant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NG1767</td>
<td>Catalase (<em>katA</em>)</td>
<td>4.00</td>
<td>5.33</td>
<td>1.54±0.22</td>
</tr>
</tbody>
</table>

*a* The "NG" gene IDs refers to the annotation of the *N. gonorrhoeae* strain FA1090 genome in the LosAlamos database (http://www.stdgen.lanl.gov/stdgen/bacteria/ngon/index.html). The "NT" ID is from the TIGR array annotation. Arrangement of the ORFs is shown in Figure 1.

*b* The ratio presented is the mean of mutant:wild-type from six replicate spots on three independent microarrays, incorporating a dye swap. Thus, the expression of each gene was measured six times. Only those genes with an expression value above two-fold and had a B statistic value above 0.0 were considered significant and included in this study. A threshold in the B statistic of 0.0 was adopted as genes with a B score>0 have a >50% probability of being truly differentially expressed. All primary data, related meta-data and a detailed summary of the protocols used in this project are available (see Experimental procedures).

*c* Microarray validation was performed using quantitative RT-PCR analysis on RNA isolated from the *N. gonorrhoeae* *oxyR* mutant and wild type strains which had been exposed to 1mM H\(_2\)O\(_2\) for 10 min prior to RNA extraction.

*d* The H\(_2\)O\(_2\)-dependence of OxyR regulation of *prx*, *gor* and *katA* in *N. gonorrhoeae* was investigated using quantitative RT-PCR on RNA isolated from the wild type strain exposed to 1 mM H\(_2\)O\(_2\) (WT+H\(_2\)O\(_2\)) or 0 mM H\(_2\)O\(_2\) (WT).

*e* The *prx* gene of *N. gonorrhoeae* is not included in the Neisseria array, however this gene is 98% identical to *prx* of *N. meningitidis* strain MC58 (NT01NG1498; NMB0946).

ND; not determined.
Table 2. Primers used in PCR and RT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S_F</td>
<td>ACGGAGGGTGCGAGCGTTAATC</td>
</tr>
<tr>
<td>16S_R</td>
<td>CTGCCTTCGCCTTGGTATTCCCT</td>
</tr>
<tr>
<td>katA_F</td>
<td>AGCCCTGCACCAAGTTACCA</td>
</tr>
<tr>
<td>katA_R</td>
<td>CAGAAGCTGTAGGTATGCGAACC</td>
</tr>
<tr>
<td>gor_F</td>
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<td>gorB</td>
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</tr>
<tr>
<td>oxyRrev</td>
<td>GACGAATCTATCCATACG</td>
</tr>
</tbody>
</table>
**Figure legends**

Figure 1. Schematic representation of (A) the *N. gonorrhoeae* genome region surrounding *prx* and *gor*, (B) the open reading frame (ORF), restriction endonuclease and (C) plasmid constructs of *prx* and *gor*, and (D) agarose gel analysis of co-transcription of *prx* and *gor* by RT-PCR. The black lines labelled Ng represent the ORF and restriction endonuclease map of a region of the *N. gonorrhoeae* genome (coordinates 910380-900833 accession number AE004969, University of Oklahoma). The open arrows above the line indicate the orientation and location of the ORFs identified in the sequence. The gene names and "NG" gene IDs are from the LosAlamos *N. gonorrhoeae* genome database (http://www.stdgen.lanl.gov/stdgen/bacteria/ngon/index.html). The "NT" ID is from the TIGR array annotation. The location of the Prx and Grx domains of *prx* is shown within the ORF. Below the ORFs the grey lines represent the plasmids constructed during this work. The vector, pGEM®-T Easy (Promega), is represented by black boxes. The restriction endonuclease sites shown indicate where the kanamycin resistance cassette (from pUC4kan; Pharmacia) was inserted. Black arrowheads indicate the primers used in this study (see Table 1). White bars represent the expected amplification products of RT-PCRs whose sizes base pairs (bp) are given in parenthesis. The agarose gel shows the products of these RT-PCR reactions using cDNA with primers for either *prx*, *gor*, or a region between these genes. A RNA sample (no reverse transcriptase (RT) added to the cDNA synthesis reaction) was used as a control for genomic DNA. Sizes of the DNA ladder are shown in bp. (E) Comparison of *prx* regulation in 1291 wild type, 1291 wild type*, and 1291oxyR::kan. The agarose gel shows the RT-PCR product for the *prx* transcript and 16S ribosomal RNA gene (indicated with arrowheads at the right of the figure).

Figure 2. Glutathione reductase (Gor) activity in *N. gonorrhoeae* wild type and gor mutant strains. Gor activity was measured in cell free extracts of overnight cultures of *N. gonorrhoeae* strain 1291 (solid line) and the gor mutant (broken line). Protein concentration in cell extracts was determined by absorbance at 280nm. Activity was determined from the decrease in [NADPH], followed as the decrease in optical density at 340 nm, using 6220 as the molar extinction coefficient. Experiments were performed in triplicate. Y-error bars indicated +/- 1 standard deviation of the mean. Experiments were conducted at least three times and data shown is a representative result. Differences between the *N. gonorrhoeae* wild type and gor strains were statistically significant (*P* values = 0.023 for 1mg protein, 0.00027 for 2 mg protein, 0.0093 for 4 mg). *P* values were computed using unpaired two-sided Student's *t* test.
Figure 3. H$_2$O$_2$ killing assay of *N. gonorrhoeae* wild type, and the *gor* and *prx* mutant strains. Cells were resuspended in BHI broth and exposed to a final concentration of 10mM H$_2$O$_2$. Experiments were performed in triplicate. Y-error bars indicate +/- 1 standard deviation of the mean. Experiments were conducted at least three times and data shown is a representative result. There is a statistically significant difference in the mean percent survival of the *prx* mutant strain relative to WT at all time points (P values ≤ 0.05 as determined using a student's T-test: P= 0.03, 15 min; P= 0.03, 30 min; P= 0.001, 60 min; P= 0.05, 75 min). There was no significant difference in the mean percent survival of the *gor* mutant strain relative to WT (P values ≥ 0.05).

Figure 4. Gonococcal association with and intracellular survival within primary human cervical epithelial (pex) cells. The histogram shows the normalised mean percent association or invasion as a function of the original inoculum of the *N. gonorrhoeae* katA, *prx*, *gor* and *oxyR* mutant strains relative to the wild type (WT). Data, determined from the number of colony forming units formed upon plating of the cervical cell lysates, were obtained from three trials performed in triplicate. Y-error bars show +/- 1 variance. There was a statistically significant difference in the mean percent survival of the *oxyR* mutant (P-values: association, 0.05; T=0, 0.005; T=1, 0.002; T=2, 0.0007) and the *gor* mutant (P-values: association, 0.08; T=0, 0.001; T=1, 0.006; T=2, 0.001) relative to *N. gonorrhoeae* strain 1291 wild type, determined using a Kruskal-Wallis non-parametric analysis of variance. The differences in the mean percent association or survival of the *katA* mutant (P-values: association, 0.16; T=0, 0.43; T=1, 0.54; T=2, 0.43) and the *prx* mutant (P-values: association, 0.93; T=0, 0.50; T=1, 0.38; T=2, 0.07) relative to *N. gonorrhoeae* strain 1291 wild type are not statistically significant.

Figure 5. Biofilm formation by *N. gonorrhoeae* strain 1291 wild type and the *oxyR*, *prx* and *gor* mutant derivatives. Panel A shows the biofilm mass over two days of growth for (1) the *N. gonorrhoeae* 1291 parent strain, and the (2) *oxyR*, (3) *prx* and (4) *gor* mutation strains. The images are stacked Z-series taken at 200x magnification. Panel B shows a COMSTAT analysis of the stack biofilm analysing the sections for biomass and the average thickness of the biofilm. The error bars represent +/- 1 standard deviation of the mean. These experiments were performed in duplicate on two different occasions and a representative result is shown. There is a statistically significant difference in the mean biomass of the *oxyR*, *prx* and *gor* mutant strain relative to WT (P values 0.016, 0.014 and 0.018, respectively, as determined using a student's T-test). There is also a statistically significant difference in the average thickness of the biofilm of the *oxyR*, *prx* and *gor* mutant strain relative to WT (P values 0.021, 0.011 and 0.007, respectively).
Figure 1
Figure 2

Figure 3
Figure 4