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Characterization of the OxyR regulon of *Neisseria gonorrhoeae*

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Summary

OxyR regulates the expression of the majority of H_2O_2 responses in Gram-negative organisms. In a previous study we reported the OxyR-dependent derepression 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 *Neisseria 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 H_2O_2 killing assays revealed increased resistance, presumably due to upregulation 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.

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Introduction

Neisseria 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 leucocytes (Archibald and Duong, 1986) which generate superoxide (O_2^-) and hydrogen peroxide (H_2O_2) as part of their bactericidal mechanism (reviewed by Hampton *et al.*, 1998; Burg and Pillinger, 2001). *N. gonorrhoeae* has evolved numerous defence mechanisms to sense and cope with this and other sources of oxidative stress that it encounters. Detoxification of H_2O_2 in *N. gonorrhoeae* depends on catalase (Tseng *et al.*, 2003; Seib *et al.*, 2004), accumulation of manganese (Mn) by the MntABC transporter (Tseng *et al.*, 2001; Seib *et al.*, 2004) and cytochrome *c* peroxidase (Ccp) (Turner *et al.*, 2003; Seib *et al.*, 2004). Other defences involved in protection against H_2O_2 include bacterioferritin (Chen and Morse, 1999) and methionine sulphoxide reductase (Taha *et al.*, 1991; Wizemann *et al.*, 1996; Skaar *et al.*, 2002).

Neisseria gonorrhoeae possesses very high constitutive levels of catalase (encoded by *katA*) (Hassett *et al.*, 1990; Zheng *et al.*, 1992) which are induced by H_2O_2 as a consequence of loss of OxyR repression (Tseng *et al.*, 2003). An *oxyR* mutant strain has ninefold higher catalase activity than constitutive levels, fourfold higher activity than maximally induced wild-type levels, and is significantly more resistant to H_2O_2 killing than the wild type (Tseng *et al.*, 2003). This is distinct from the situation in *Escherichia coli* and *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 H_2O_2 is seen in *oxyR* mutant strains (Christman *et al.*,

1985; 1989). OxyR of *N. 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 *E. coli*. In addition, *N. gonorrhoeae* OxyR can complement an *E. coli 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 *E. coli* (reviewed by Storz and Imlay, 1999; Pomposiello and Demple, 2001). OxyR regulates expression of the majority of H_2O_2 responsive genes in *E. coli*, including *katG* (hydroperoxidase I), *ahpCF* (alkylhydroperoxide reductase), *gorA* (glutathione reductase), *grxA* (glutaredoxin 1), *trxC* (thioredoxin 2), *fur* (repressor of iron uptake), *dps* (unspecific DNA binding protein), *oxyS* (regulatory RNA), *dsbG* (disulphide bond chaperone-isomerase) and *fhuF* (protein required for iron uptake), *hemH* (haem biosynthetic gene), six-gene *suf* operon (may participate in Fe-S cluster assembly or repair), and *uxuA* (mannonate hydrolase) (Pomposiello and Demple, 2001; Zheng *et al*., 2001a,b). These OxyR-regulated genes have direct (e.g. removal of H_2O_2 by *katG* and *ahpC*; control of redox balance by *gor*, *grxA* and *trxC*) and indirect (e.g. control of the *fur* and *oxyS* regulators that affect numerous other genes) roles in defences against oxidative stress. The OxyR regulon of *E. coli* was determined, in part, via DNA microarray-mediated transcription profiling of the H_2O_2 response (after exposure to 1 mM H_2O_2 for 10 min) of an *E. coli* wild-type strain relative to an *oxyR* mutant strain (Zheng *et al*., 2001b).

OxyR is constitutively expressed in *E. coli* and *S. typhimurium* (Storz *et al*., 1990; Zheng *et al*., 1998). H_2O_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 disulphide bond is then reduced by glutaredoxin 1 (GrxA) and glutathione (γ-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 (> 45 bp) with limited sequence similarity. Both the oxidized 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

Characterization of the OxyR regulon of N. gonorrhoeae: *DNA microarray analysis and real-time 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 1291*oxyR::kan* mutant strain (Tseng *et al*., 2003) was compared by analysis on *N. 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, 1291*oxyR::kan*, which may confuse interpretation of microarray analysis, we constructed a wild-type *oxyR* 'knock-in' version of the 1291*oxyR::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 1291 wild type* strains were compared with the parental 1291 strain and with 1291*oxyR::kan* to confirm that the 1291*oxyR::kan* H_2O_2 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 1291*oxyR::kan* phenotype, but that the 1291*oxyR::kan* phenotype was solely due to inactivation of the *oxyR* gene.

Total RNA was isolated from wild-type and 1291*oxyR::kan* mutant strain cultures that had been grown to exponential phase then exposed to 1 mM H_2O_2 for 10 min. Overall, three genes were differentially regulated by greater than twofold (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 Prx and a putative Gor respectively, were downregulated in the *oxyR* mutant strain relative to wild type (Table 1). The gene encoding catalase, *katA* (NG1767), was upregulated 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 characterized in *N. gonorrhoeae* and are the focus of this study (see Fig. 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_2O_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

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

Gene ID[a]	Description	Microarray[b]		Microarray validation[c]	H_2O_2 regulation[d]
		oxyR : WT	B-Stat	*oxyR* : WT	WT + H_2O_2 : WT
Reduced expression in *oxyR* mutant					
NG0925	Glutathione reductase (*gor*)	0.32	6.24	0.11 ± 0.015	8.05 ± 0.78
NT01NG1498	ORF (5′ end of *gor*)	0.35	6.38	ND	ND
NG0926	Peroxiredoxin (*prx*)[e]	0.31	6.10	0.11 ± 0.004	6.40 ± 0.15
Increased expression in *oxyR* mutant					
NG1767	Catalase (*katA*)	4.00	5.33	1.54 ± 0.22	4.65 ± 0.67

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 Fig. 1.
b. The ratio presented is the mean of mutant : wild type (WT) 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 twofold 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 WT strains which had been exposed to 1 mM H_2O_2 for 10 min prior to RNA extraction.
d. The H_2O_2 dependence of OxyR regulation of *prx*, *gor* and *katA* in *N. gonorrhoeae* was investigated using quantitative RT-PCR on RNA isolated from the WT strain exposed to 1 mM H_2O_2 (WT + H_2O_2) or 0 mM H_2O_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.

(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 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 hypothesized 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 organized 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 (*dLdH*, 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-sulphur 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_2O_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). To investigate the H_2O_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_2O_2. H_2O_2 induced expression of *prx* (6.4-fold), *gor* (eightfold) 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_2O_2. On the other hand, OxyR derepresses catalase expression upon exposure to increased levels of H_2O_2. This supports

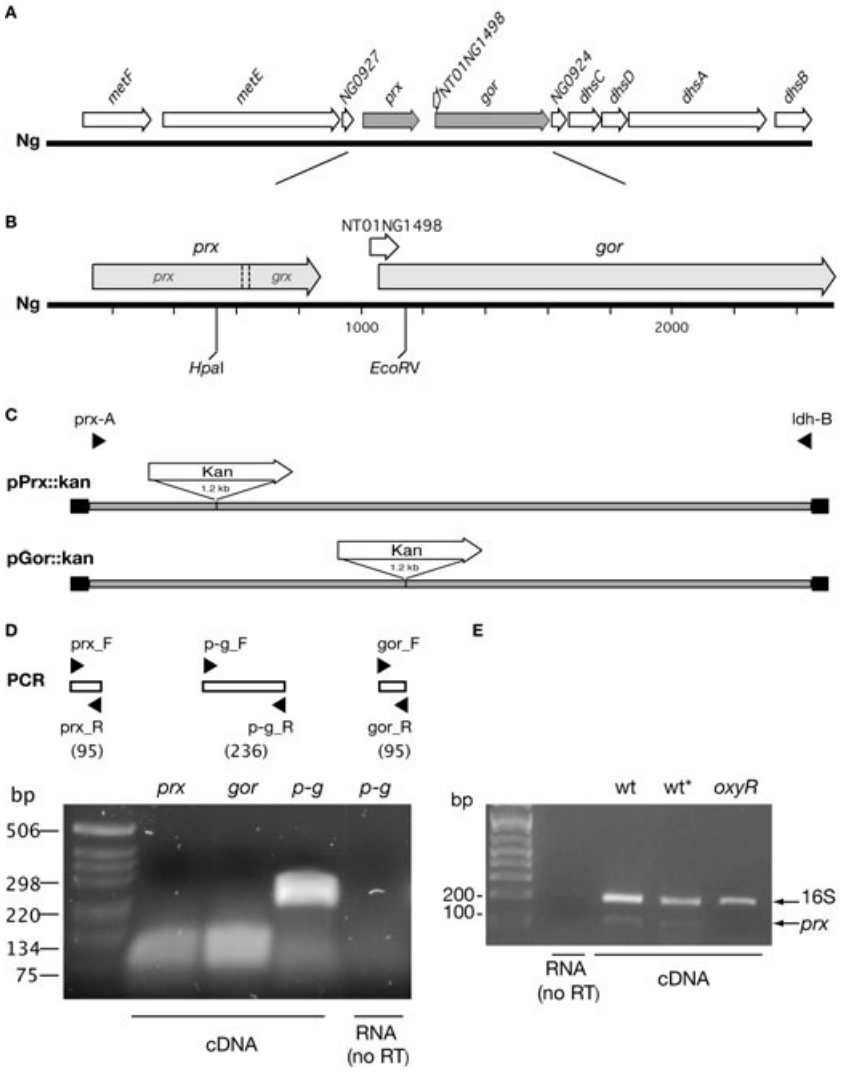

Fig. 1. Schematic representation of (A) the *N. gonorrhoeae* genome region surrounding *prx* and *gor*, (B) the ORF and restriction endonuclease map (C) the plasmid constructs of *prx* and *gor*, and (D) agarose gel analysis of co-transcription of *prx* and *gor* by RT-PCR. The black lines labelled Ng represent the ORF and restriction endonuclease map of a region of the *N. gonorrhoeae* genome (co-ordinates 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 2). 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 (WT), 1291 wild type* (WT*) and 1291*oxyR::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).

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 co-transcription (see Fig. 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 Fig. 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 1291*oxyR::kan* (see Fig. 1E). The *prx-gor* transcript, reduced in the 1291*oxyR::kan* mutant returned to 1291 wild type levels in the 1291 wild type* strain, further confirming that the 1291*oxyR::kan* regulatory phenotype was not due to second site suppressor mutations.

Table 2. Primers used in PCR and RT-PCR.

Primer	Sequence (5′–3′)
16S_F	ACGGAGGGTGCGAGCGTTAATC
16S_R	CTGCCTTCGCCTTCGGTATTCCT
katA_F	AGCCCTGCACCAAGTTACCA
katA_R	CAGAAGCTGTAGGTATGCGAACC
gor_F	GATGTGGAAGAATGGCCTGC
gor_R	CGCAATTTGGATATGGTCGTC
prx_F	ACGGCGAATTTACCGAAGGTA
prx_R	CGTCGTTAACCAGCATGGAGTA
prxA	TGGCTTTGCAAGATCGTACC
gorB	AACGGCATATCCAGCATTTG
preprx	GCGATTCACAATTATTTCTCAAACC
prx-gor_F	GAAGATTTGGAAGCTTACTTGG
prx-gor_R	ACATATCGGTTTCAGACAGC
oxyRfor	CGGAGAACCGGTCATCCA
oxyRrev	GACGAATCTATCCATACG

Construction of N. gonorrhoeae prx *and* gor *mutant strains*

To investigate the role of Prx and Gor in *N. gonorrhoeae*, *prx* and *gor* mutant strains were constructed via insertion of a kanamycin-resistance cassette into the open reading frame (ORF) of the *prx* and *gor* genes of *N. gonorrhoeae* strain 1291 (Fig. 1C). These mutant strains were confirmed by PCR analysis with the primers preprx and prx_R for the *prx* mutant strain or preprx and gor_R for the *gor* mutant strain (Table 2). The growth characteristics of the *N. gonorrhoeae* wild-type and the *prx*, *gor* and *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 *N. gonorrhoeae* wild-type, *gor* and *prx* mutant strains. Gor catalyses the reaction $2GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$. 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 (Fig. 2) and increasing GSSG concentration (data not shown). No significant Gor activity was present in the *gor* mutant strain relative to the wild-type strain (see Fig. 2). These results indicate that NG0925 does encode the Gor of *N. gonorrhoeae.* Gor activity in the *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 *gor* mutant strain is not a result of a polar effect from the *prx* mutation.

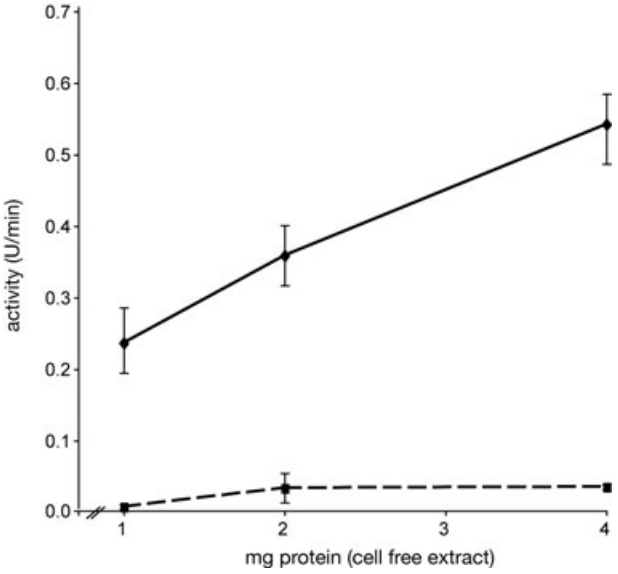

Fig. 2. Glutathione reductase activity in *N. gonorrhoeae* wild-type and *gor* mutant strains. Gor activity was measured in cell free extracts of overnight cultures of *N. gonorrhoeae* strain 1291 (solid line) and the *gor* mutant (broken line). Protein concentration in cell extracts was determined by absorbance at 280 nm. 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 1 mg protein, 0.00027 for 2 mg protein, 0.0093 for 4 mg). *P*-values were computed using unpaired two-sided Student's *t*-test.

The apparent K_m ($K_{m\ app}$) for the reduction of GSSG by *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 *oxyR* mutant of *N. gonorrhoeae* is highly resistant to H_2O_2 stress (Tseng *et al.*, 2003). This resistance is presumably in part due to the increased catalase activity seen in the *oxyR* mutant strain (Tseng *et al.*, 2003); a *katA* mutant of *N. gonorrhoeae* is highly sensitive to H_2O_2 (Seib *et al.*, 2004). To investigate the role of the two other OxyR-regulated proteins in the oxidative stress response, H_2O_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

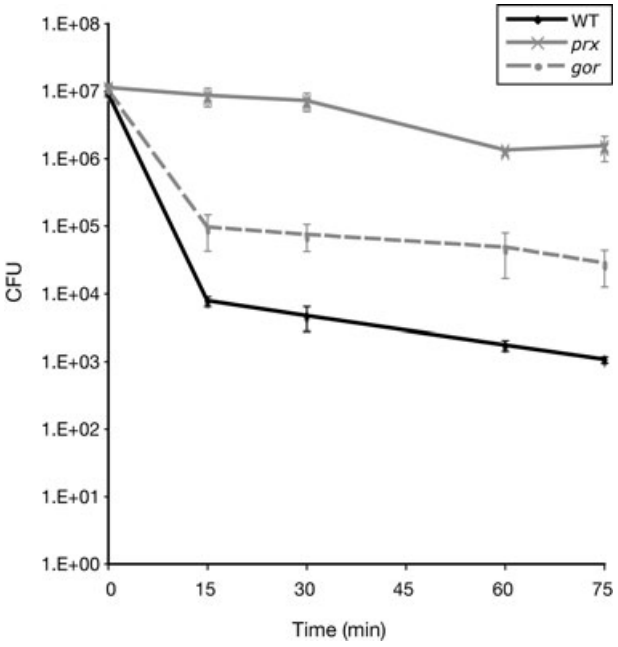

Fig. 3. H_2O_2 killing assay of *N. gonorrhoeae* wild type (WT), and the *gor* and *prx* mutant strains. Cells were resuspended in BHI broth and exposed to a final concentration of 10 mM H_2O_2. Experiments were performed in triplicate. Y-error bars indicate ±1 standard deviation of the mean. Experiments were conducted at least three times and data shown are a representative result. There is a statistically significant difference in the mean per cent 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 per cent survival of the *gor* mutant strain relative to WT (*P*-values ≥ 0.05).

killing with H_2O_2 than the wild-type strain ($P \leq 0.05$; Fig. 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 H_2O_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 with wild-type gonococci. The *oxyR* and *gor* mutants showed a more significant decrease in invasion and survival over 2 h, relative to wild type (Fig. 4). However, there was no statistically significant difference in the mean per cent association or survival of the *katA* and *prx* mutant strains relative to *N. gonorrhoeae* strain 1291 wild type (Fig. 4).

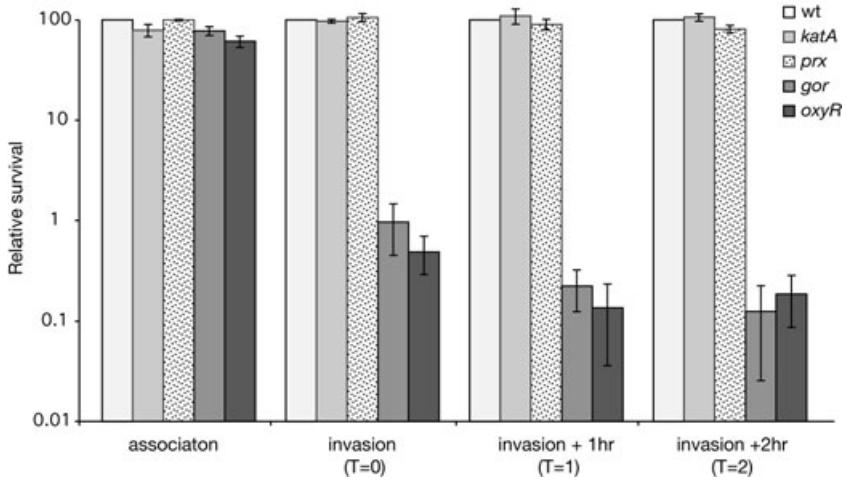

Fig. 4. Gonococcal association with and intracellular survival within primary human cervical epithelial (pex) cells. The histogram shows the normalized mean per cent association or invasion as a function of the original inoculum of the *N. gonorrhoeae katA*, *prx*, *gor* and *oxyR* mutant strains relative 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 per cent 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 per cent 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.

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 2 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 (Fig. 5).

Discussion

Neisseria gonorrhoeae encounters significant levels of ROS, including H_2O_2, within the female urogenital tract as a result of exposure to resident lactic acid bacteria (Whittenbury, 1964) and activated polymorphonuclear leucocytes (Archibald and Duong, 1986). The peroxide stress response of *N. gonorrhoeae* is unusual in that it contains two H_2O_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 uncharacterized proteins of *N. gonorrhoeae*, Prx and Gor. All three OxyR regulated genes are upregulated in response to H_2O_2 stress, indicating that they play a role in protecting *N. gonorrhoeae* from damage caused by H_2O_2.

OxyR regulates more than 10 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_2O_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_2O_2 stress (Tseng *et al*., 2003), while sensitivity to H_2O_2 is seen in an *oxyR* mutant of *E. coli* (Christman *et al*., 1985; 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-haem 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 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 co-ordinately regulated in *N. gonorrhoeae* is appropriate in light of their co-ordinated function. The Prx protein identified in *N. gonorrhoeae* is 98% identical to the hybrid Prx (N-terminus Prx domain and C-terminus Grx domain) characterized in *N. meningitidis* which reduces various peroxides, including H_2O_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 Fig. 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 sulphenic acid by the peroxide substrate. The mechanism by which the thiol is regenerated from the sulphenic 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 disulphide 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_2O_2, *tert*-butylhydroperoxide and cumene hydroperoxide (Vergauwen *et al*., 2001; 2003; Cha *et al*., 2004; Pauwels

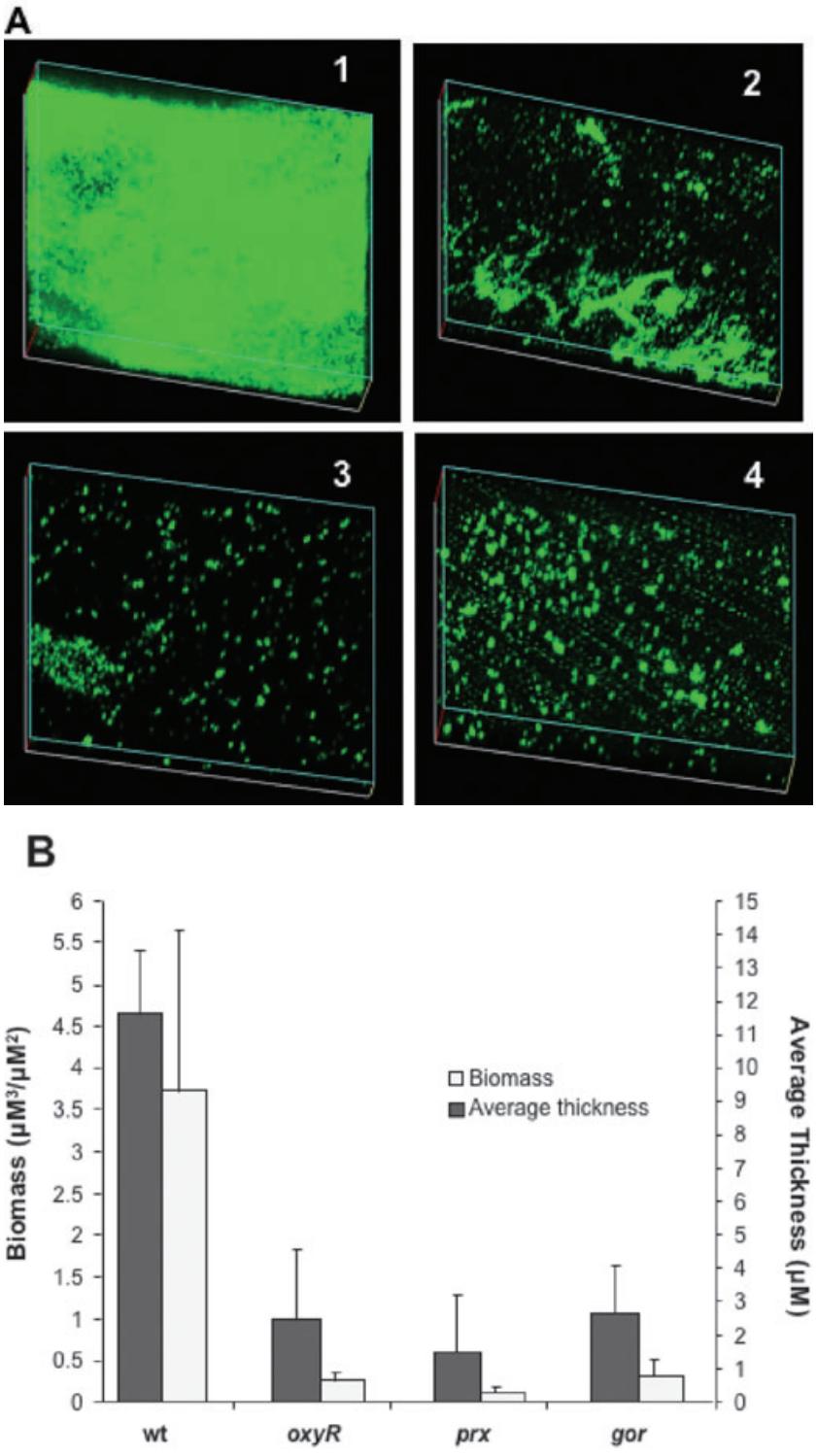

Fig. 5. Biofilm formation by *N. gonorrhoeae* strain 1291 wild type and the *oxyR*, *prx* and *gor* mutant derivatives. Panel A shows the biofilm mass over 2 days of growth for (1) the *N. gonorrhoeae* 1291 parent strain, and the (2) *oxyR*, (3) *prx* and (4) *gor* mutation strains. The images are stacked z-series taken at 200× 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 strains 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 strains relative to WT (*P*-values 0.021, 0.011 and 0.007 respectively).

et al., 2004). 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_2O_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_2O_2 (Pauwels *et al*., 2004), results in increased H_2O_2 levels that cause derepression of catalase in *N. gonorrhoeae*.

Gor (NAD(P)H:oxidized-glutathione oxidoreductase) is nearly ubiquitous and has been well characterized 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 (GSH) 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 (5 mM 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 $\pm$ 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 (Kunert *et al*., 1990; Becker-Hapak and Eisenstark, 1995). 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 as 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 (Schmidt and Walter, 1994; Rochelle *et al*., 1998; Battistoni *et al*., 2000). 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 with 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 with 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 with 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_2O_2; Ag43-mediated cell aggregation is believed to confer protection against H_2O_2 killing (Schembri *et al*., 2003). A similar situation is seen in *B. pseudomallei*, where *oxyR* mutant strains are hypersensitive to H_2O_2 and paraquat and have increased biofilm formation in minimal medium (Loprasert *et al*., 2002). Oxidative stress defences are also induced during immobilized 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 result in modification of the host environment which induces 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

Neisseria 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 BHI agar or broth (Acumedia) supplemented with 10% Levinthal's base (Alexander 1965) and 1% IsoVitaleX (Becton Dickinson) at 37°C in 5% CO_2. *E. coli* strain DH5α was cultured in Luria–Bertani (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*. (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.

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 kanaymcin-cassette-containing fragment derived from pUC4Kan (see Fig. 1). These constructs were linearized 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 1291*oxyR::kan*, we constructed a wild-type *oxyR* 'knock-in' version 'wild type*' of the 1291*oxyR::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 linearized pHJT*oxyR* (Tseng *et al*., 2003), which contains the wild-type *oxyR* gene, into the 1291*oxyR::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 1291 wild type* strains were analysed by comparison with the parental wild-type 1291 strain and to 1291*oxyR::kan*, to confirm that both the 1291*oxyR::kan* H_2O_2 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 1291*oxyR::kan* phenotype, but that the 1291*oxyR::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 = 0.2–0.5). These cultures were then exposed to 1 mM 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 microfluidic analysis on a bioanalyser (Agilent Technologies).

All microarray analysis was performed on *N. gonorrhoeae/meningitidis* genome arrays (TIgr; http://pfgrc.tigr.org/

slide_html/array-descriptions/N_gonorrhoeae_2.shtml). Each microarray consists of 6389 70mer oligonucleotides representing 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.

Five micrograms of each total RNA sample was labelled using random hexamers and direct incorporation of fluorescently Cy3- or Cy5-labelled nucleotides as previously described (Grimmond *et al*., 2000). The hybridizations were performed in triplicate and incorporated a dye-swap to account for dye bias. After 16 h of hybridization, the arrays were washed and scanned on an Agilent G2565BA microarray scanner at a 5 micron resolution. The resulting images of the hybridizations were analysed using Imagene 5.5 (BioDiscovery) and the mean foreground, mean background and spot/signal quality determined.

All primary data were 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 normalization, 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 are 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 RT-PCR

Total RNA was isolated by using the RNeasy kit (Qiagen) as described above. Cultures were exposed to 1 mM 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 RT-PCR reactions were performed in triplicate in a 25 μl mixture containing cDNA (5 μl of 1/5 dilution), 1× 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 were 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 $2^{\Delta\Delta CT}$ relative quantification method. To investigate H_2O_2 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 H_2O_2.

Semi-quantitative RT-PCR

PCR was carried out in 50 μl reactions using 1× Taq buffer, 1.5 mM $MgCl_2$, 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 30 s, 72°C for 30 s and one 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 280 nm. The assay mixture included 50 mM K_2HPO_4/0.1 mM EDTA pH 7.5, 100 μM NADPH, 1 mM 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 H_2O_2 (Johnson *et al*., 1993) killing assays were performed using established methods as described by Tseng *et al*. (2001). Briefly, cells from agar plates were harvested, resuspended in PBS and 10^5–10^7 cells were added to a solution of BHI broth to a final volume of 100 μl. The killing assay was started by the addition of a final concentration either 10 mM paraquat (Sigma), or 4.3 mM xanthine and 300 mU ml^{-1} xanthine oxidase (Sigma), or 10 mM H_2O_2 (Riedel-de Haen). Cultures were incubated at 37°C/5% CO_2 and at various time points samples were taken, plated onto BHI agar after serial dilutions and incubated at 37°C in 5% CO_2. 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 cell survival assay

Primary human ectocervical epithelial 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% CO_2. 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 1 h or 2 h incubation. Following each assay, pex cells were lysed with 0.5% saponin to release invasive bacteria, and serial dilutions were plated to determine colony-forming units (cfu). The per cent 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^{-1} IsoVitaleX (Becton-Dickinson, Franklin Lakes, NJ), and incubated at 37°C and 5% CO_2. Overnight plate cultures were used to create cell suspensions for inoculation of biofilm flow chambers.

Neisseria 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^{-1} chloramphenicol to maintain pGFP. Cell suspensions of 2×10^8 cfu ml^{-1} (in approximately 1 ml of biofilm media) were used to inoculate $37 \times 5 \times 5$ mm flow cell chamber wells. These chambers were designed to reduce fluid sheer on biofilm (versus typical 1 mm depth wells). Flow chambers were incubated under static conditions at 37°C for 1 h post inoculation. Chambers were then incubated for another 48 h under 180 μl min^{-1} flow. After 48 h, 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, 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 eight-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.

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