

The transcriptome response of *Neisseria gonorrhoeae* to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection

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Summary

Symptomatic gonococcal infection, caused by the pathogen *Neisseria gonorrhoeae* (Gc), is characterized by the influx of polymorphonuclear leukocytes (PMNs) to the site of infection. Although PMNs possess several mechanisms of oxidative killing, intact Gc can be found associated with PMNs, suggesting that gonococcal defences against oxidative stress are crucial for its ability to evade killing by PMNs. We used microarrays to identify genes that were differentially expressed after transient exposure of Gc to hydrogen peroxide (H_2O_2). Of the 75 genes found to be upregulated after H_2O_2 treatment, over one-quarter, including two of the most highly upregulated genes (NGO1686 and NGO554), were predicted to encode proteins with unknown functions. Further characterization of a subset of these upregulated genes demonstrated that NGO1686, a putative zinc metalloprotease, protects against oxidative damage caused by both H_2O_2 and cumene hydroperoxide, and that NGO554, a Gc-specific protein, acts to protect against damage caused by high levels of H_2O_2 . Our current study also ascribes a role in H_2O_2 damage protection to *recN*, a gene previously characterized for its role in DNA repair. A PMN survival assay demonstrated that the *recN* and NGO1686 mutants were more susceptible to killing than the parent strain FA1090. These results define for the first time the robust transcriptional response to H_2O_2 by this strict human pathogen and underscore the importance of this system for survival to host defences.

Introduction

All aerobically grown cells are exposed to toxic reactive oxygen species (ROS) that are evolved via the process of oxidative phosphorylation during normal cellular respiration. These ROS include hydrogen peroxide (H_2O_2), hydroxyl radical (HO^*) and superoxide anion ($O_2^{\cdot-}$), and are capable of damaging many different biomolecules, including proteins, membrane lipids, carbohydrates and nucleotide bases, through a variety of different reactions (Fang, 2004). *Neisseria gonorrhoeae* (Gc), the only causative agent of the disease gonorrhoea, is an obligate human pathogen that colonizes mucosal surfaces, most commonly those of the urogenital tract. In addition to self-generated ROS, Gc is also exposed to ROS during infection. In the human body, H_2O_2 -producing commensal lactobacilli inhabit the same niche as Gc, and these lactobacilli may inhibit the growth of Gc (Zheng *et al.*, 1994; St Amant *et al.*, 2002). The hallmark of symptomatic gonococcal infection is a massive influx of activated polymorphonuclear leukocytes (PMNs) into the urethra resulting in a purulent discharge (Shafer and Rest, 1989). PMNs kill microorganisms through the combined activity of antimicrobial proteins and ROS (Segal, 2005). PMNs are capable of delivering a potent bactericidal burst, generating substantial amounts of ROS; however, viable Gc can be found associated with PMNs both bound to the cell surface and intracellularly, suggesting that PMNs may be unable to kill Gc (Shafer and Rest, 1989; Simons *et al.*, 2005). Thus, the ability of Gc to avoid the PMN-derived oxidative burst and subsequent killing may be an important virulence factor, and several gonococcal genes have been identified that protect against oxidative damage. The catalase (*kat*) (Soler-Garcia and Jerse, 2004), peptide methionine sulphoxide reductase (*msrAB*) (Skaar *et al.*, 2002a), cytochrome *c* peroxidase (*ccp*) (Turner *et al.*, 2003; Seib *et al.*, 2004), bacterioferritin (*bfrA*) (Chen and Morse, 1999) and cytochrome *c* oxidase (*sco*) (Seib *et al.*, 2003) genes, and an Mn(II) uptake system (*mntC*) (Tseng *et al.*, 2001) all encode products that offer various degrees of protection against different types of oxidative damaging agents *in vitro*. Thus, Gc is exposed to both internally and externally generated sources of oxidative stress and is likely to employ a number of mechanisms to protect itself from oxidative damage.

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Study of the proteome and transcriptome response of bacteria to oxidative damage was begun nearly 20 years ago when the induction of approximately 30 *Salmonella* proteins after H₂O₂ treatment was observed by two-dimensional (2-D) gel electrophoresis (Christman *et al.*, 1985; Morgan *et al.*, 1986). Further characterization of this response has revealed that the positive transcriptional regulators SoxR and OxyR sense O₂⁻ and H₂O₂ stress, respectively, and co-ordinate the expression of a number of antioxidant genes (Storz and Imlay, 1999). Recently, microarray analyses have been employed to study gene expression in response to oxidative damage in several different bacteria (Zheng *et al.*, 2001; Porwollik *et al.*, 2003; Mostertz *et al.*, 2004; Palma *et al.*, 2004). These studies have revealed that, in addition to those genes with defined antioxidant functions, many encode proteins with no established function. Studies of the pathogenic *Neisseria* suggest that their response to oxidative damage is fundamentally different from that of *Escherichia coli*. First, the OxyR protein of Gc acts as a repressor of catalase (*kat*), which is the opposite of the *E. coli* paradigm (Tseng *et al.*, 2003). Second, studies of the response to oxidative damage in *Neisseria meningitidis* (Nm) revealed a response that differs from that which has been observed in other bacterial species (Grifantini *et al.*, 2004).

In this study we used *Neisseria* microarrays to detect alterations in global gene expression by Gc in response to the oxidative damaging agent H₂O₂. We demonstrate a significant regulation of > 150 genes in response to oxidative challenge. Several of the upregulated genes have been previously shown to be important for protection against oxidative damage in Gc; however, many of the upregulated genes encode proteins with either no demonstrated function or with an untested role in oxidative damage protection. To investigate the roles of some of these gene products, we created deletion mutants in two genes with no established function in Gc and studied their roles, as well as that of *recN*, in protection against oxidative killing. All three genes encode proteins that protected Gc against oxidative killing by H₂O₂. Furthermore, we demonstrated that mutants in *recN* and NGO1686 show greater susceptibility to killing by PMNs. Thus, this study illustrates the utility of microarrays for both elucidating the antioxidant response of Gc and uncovering potential novel virulence factors.

Results

Transcriptional changes induced by H₂O₂

To begin to understand how Gc responds to oxidative stress, we mapped the global transcriptional response of Gc to H₂O₂. We used microarrays to measure changes in

steady-state levels of Gc strain FA1090 mRNA resulting from a 15 min treatment with 5 mM H₂O₂. This length of exposure was predicted to be sufficient to detect changes in mRNA levels induced by treatment based on a doubling time of 45–60 min for Gc. As the main source of variability in microarray experiments is biological variability (Lee *et al.*, 2000; J.K. Davies *et al.*, unpubl. data), we performed four independent biological replicates by growing and treating cultures with H₂O₂ on four separate days. Pairs of differentially labelled untreated and H₂O₂-treated cDNAs from each biological replicate were hybridized to two microarray slides, containing each gene spotted in triplicate: for one slide the untreated cDNA was labelled with Alexa555 and the H₂O₂-treated was labelled with Alexa647, and for the second slide the fluorescent labels were reversed (dye-swap). Thus, a maximum of 24 data points was averaged to determine changes in gene expression as a result of H₂O₂ treatment. Seventy-five genes were upregulated ≥ 2.5 -fold, with *P*-values ≤ 0.01 by the Student's *t*-test and representation in at least six of eight slides (microarray annotation supplied by J.K. Davies *et al.*, unpubl. data) (Table 1). Eighty genes were downregulated by the same criteria and are included in *Supplementary material* (Table S1). Many of the downregulated genes encode functions related to transcription and translation, which has been previously observed (Mostertz *et al.*, 2004), but the only downregulated genes that will be discussed in detail are three putative transcriptional regulators. Genes with predicted functions particularly germane to oxidative damage and the regulation of the response to oxidative damage are detailed below.

Oxidative response/repair genes

Peroxide treatment led to the upregulation of seven genes whose products are predicted or known to be involved in protecting against oxidative damage (Table 1). In Gc, mutations in the *ccp* (Turner *et al.*, 2003; Seib *et al.*, 2004) and *msrAB* (Skaar *et al.*, 2002a) genes result in decreased survival to oxidative damaging agents. *ccp* encodes a cytochrome *c* peroxidase and catalyses the reduction of H₂O₂ to water; *msrAB* encodes a peptide methionine sulphoxide reductase enzyme which catalyses the reduction of damaged (oxidized) methionine residues on proteins. Thioredoxins and glutaredoxins function in maintaining a reduced cytosol, which is essential for the control of protein function (Carmel-Harel and Storz, 2000), and the *ald* gene, predicted to encode alcohol dehydrogenase, also shows sequence similarity to Zn-dependent oxidoreductases (as annotated in STDGEN; <http://www.stdgen.lanl.gov/>). Finally, we observed upregulation of the superoxide dismutase gene (*sodB*), whose product catalyses the dismutation of superoxide into H₂O₂. In *E. coli*, the *sodB* gene has a minor role in protection against

oxidative stress (Kargalioglu and Imlay, 1994); however, a Gc *sodB* mutant has no diminished resistance to paraquat or xanthine/xanthine oxidase (Tseng *et al.*, 2001). Thus, we observed upregulation of many genes whose products have been demonstrated to function as antioxidants in bacteria.

Heat shock genes

We observed induction of a number of heat shock genes

and molecular chaperones after H₂O₂ treatment (*groEL*, *groES*, *grpE*, *dnaJ*, *dnaK*, *clpB*, *lonC*, *ftsH* and *secB*). Overlap of the heat shock regulon with other stresses, including H₂O₂, was originally observed through 2-D gel analysis of proteins in *Salmonella typhimurium* (Morgan *et al.*, 1986) and has been repeatedly shown through transcriptional genomic analysis (Zheng *et al.*, 2001; Mostertz *et al.*, 2004; Palma *et al.*, 2004). This suggests that the genes encoding these proteins respond to multiple stimuli.

Table 1. Genes upregulated in response to hydrogen peroxide.

Proposed function	Gene name	Fold ^a change	ORF ID ^b
Oxidative response/repair			
Thioredoxin	<i>trxA</i>	13.5	NGO652
Glutaredoxin	<i>grxC</i>	4.8	NGO114 ^{c,d}
Peptide methionine sulfoxide reductase	<i>msrAB</i>	3.7	NGO2059
Zinc-binding alcohol dehydrogenase	<i>ald</i>	3.1	NGO186
Glutaredoxin	<i>grxB</i>	3.0	NGO1381
Superoxide dismutase	<i>sodB</i>	2.8	NGO0450
Cytochrome C551 peroxidase	<i>cpx; ccp</i>	2.7	NGO1769
Iron responsive/iron related			
Iron-regulated outer membrane protein	<i>fetA</i>	16.8	NGO2093
Transferrin-binding protein 2	<i>tbp2</i>	15.7	NGO1496 ^c
Transferrin-binding protein 1	<i>tbp1</i>	10.8	NGO1495 ^c
Haem utilization protein	<i>pigA</i>	9.8	NGO1318
Fumarate hydratase	<i>fumC</i>	8.4	NGO1029
Transport	<i>tonB</i>	7.6	NGO1379 ^c
Ferric-uptake regulator	<i>fur</i>	6.7	NGO1779
Periplasmic iron-binding transporter	<i>tbpA</i>	5.5	NGO217
Periplasmic iron-binding transporter	<i>fetB</i>	4.7	NGO2092
Fe-S cluster assembly	<i>nifU</i>	4.6	NGO633
Aminotransferase/Fe-S cluster assembly	<i>nifS</i>	4.1	NGO0636 ^{c,e}
DNA repair	<i>recN</i>	3.0	NGO318^f
Haemoglobin-haptoglobin utilization	<i>hpuA</i>	2.6	NGO2110
Biopolymer transport	<i>exbB</i>	4.7	NGO1378 ^c
Biopolymer transport	<i>exbD</i>	4.4	NGO1377 ^c
Energy/intermediary metabolism			
Methylcitrate synthase	<i>prpC</i>	5.6	NGO1525
Dihydrolipoamide dehydrogenase	<i>dLdH</i>	4.9	NGO925
S-adenosylmethionine synthetase	<i>metK</i>	3.9	NGO106
Aldehyde dehydrogenase	<i>aldA</i>	3.7	NGO2114
Phosphoglycolate phosphatase	<i>gph</i>	3.5	NGO1052
Phosphoglycerate mutase	<i>gpm</i>	2.8	NGO1258
Pyruvate kinase	<i>pykA</i>	2.7	NGO1881
Heat shock response/chaperones/protein modification			
Chaperone	<i>clpB</i>	23.8	NGO1046
Chaperone	<i>dnaK</i>	10.8	NGO1429
Protein export	<i>secB</i>	6.9	NGO116 ^{c,d}
Peptidyl-prolyl <i>cis-trans</i> isomerase	<i>ppiB</i>	5.9	NGO376
Heat shock protein	<i>grpE</i>	5.8	NGO1422
Lon protease	<i>lonC</i>	4.5	NGO775
Chaperone	<i>groES</i>	4.1	NGO2094
Cell division, protein degradation	<i>ftsH</i>	3.1	NGO382
Chaperone	<i>dnaJ</i>	3.0	NGO1901
Chaperone	<i>groEL</i>	2.6	NGO2095
Regulators			
Repressor protein		6.3	NGO1427
Transcriptional regulator, AraC family		5.1	NGO2115
probable transcriptional regulator		2.5	NGO637 ^{c,e}
Cell surface structures			
Lipid biosynthesis	<i>lpxC</i>	10.5	NGO2065
Phosphoglucomutase	<i>pgm</i>	3.0	NGO375
Putative adhesin complex protein	<i>lecA</i>	2.5	NGO1981
Phosphatidylserine decarboxylase	<i>psd</i>	2.7	NGO1206

Table 1. cont.

Proposed function	Gene name	Fold ^a change	ORF ID ^b
DNA replication/recombination/repair DNA helicase	<i>dnaB</i>	6.5	NGO1110 or NGO485 ^g
Synthesis of cofactors, prosthetic groups, cofactors Geranyltransferase	<i>ispA</i>	3.2	NGO1735
Nucleotide biosynthesis and metabolism Adenylate kinase	<i>adk</i>	3.0	NGO400
Guanylate kinase	<i>gmk</i>	2.5	NGO1310
Thiamin binding and transport	<i>tbpA</i>	2.5	NGO2056 ^{c,h}
Amino acid biosynthesis Isopropylmalate synthase	<i>leuA</i>	3.3	NGO848
Para-aminobenzoate synthase glutamine Amidotransferase	<i>trpG</i>	3.1	NGO1204 ^e
Anthranilate phosphoribosyltransferase	<i>trpD</i>	3.1	NGO1203 ^e
Unknown role Acyl CoA thioester hydrolase		16.1	NGO1055
Hypothetical proteins Gc-specific hypothetical protein (HP)		70.6	NGO554^f
Conserved HP (putative metalloendopeptidase)		19.3	NGO1686^f
<i>Neisseria</i> -specific HP		6.8	NGO108
Conserved HP		6.7	NGO1948 ^e
<i>Neisseria</i> -specific HP		5.8	NGO1771
Conserved HP		5.0	NGO1716
Conserved HP		4.6	NGO555
<i>Neisseria</i> -specific HP		4.2	NGO865
Gc-specific HP		4.2	NGO2142
Conserved HP		4.2	NGO1426
Gc-specific HP		3.9	NGO1947 ^e
<i>Neisseria</i> -specific HP		3.9	NGO1428
<i>Neisseria</i> -specific HP		3.2	NGO322
Conserved HP		3.0	NGO1900
<i>Neisseria</i> -specific HP		2.9	NGO1690
Conserved HP		2.9	NGO634
Conserved HP		2.7	NGO1174
Conserved HP		2.6	NGO387
Protein similar to phage proteins		2.5	NGO1197
Conserved HP		2.5	NGO1056

a. Fold change represents the ratio of mRNA transcript levels in hydrogen peroxide-treated strain FA1090 to untreated strain FA1090.

b. Open reading frame (ORF) ID is as annotated in STDGEN (<http://www.stdgen.lanl.gov/>).

c. Indicates that a neighbouring gene(s) is also upregulated and oriented in the same transcriptional direction, with a <100 nucleotide intergenic region, suggesting that the genes are transcriptionally linked.

d. NGO114 and NGO116 are neighbouring genes; NGO115 overlaps NGO116 in the opposite transcriptional orientation.

e. NGO636 and NGO637 are likely to be transcriptionally linked to NGO635, which showed a 5.8-fold upregulation ($P = 0.012$), but was only represented in five-eighths slides, and was therefore not included in our list.

f. Genes in bold were insertionally inactivated and studied for their roles in protection against oxidative damage.

g. There are two identical copies of *dnaB* in the FA1090 genome.

h. NGO2056 is likely to be transcriptionally linked to NGO2057, which showed a 2.1-fold upregulation ($P = 0.0008$), and was therefore not included in our list.

The data presented above represent the average of four biological replicates and eight slides.

Iron-related and Fur-regulated genes

Fifteen genes that are related to iron acquisition (Perkins-Balding *et al.*, 2004), or are known to be regulated by Fur in Gc (Sebastian *et al.*, 2002), were upregulated by treatment with H₂O₂. The majority of the products of these genes (*tbpA*, *tbpB*, *frpB*, *fbpA*, *fetB*, *exbB*, *exbD*, *hpuA*, *tonB*, *pigA* and *fur*) are involved in iron acquisition. The *fumC*, *nifS* and *nifU* genes encode the tricarboxylic acid (TCA) cycle enzyme fumarate hydratase and two proteins involved in the synthesis of Fe-S clusters respectively. The Fur-regulated (Sebastian *et al.*, 2002) *recN* gene is

involved in the repair of damaged DNA in both *E. coli* (Picksley *et al.*, 1984) and Gc (Skaar *et al.*, 2002b), but its exact biochemical function is unknown. Although it is unclear exactly why *recN* transcription increases as a result of exposure to H₂O₂, this has been observed in other bacteria (Zheng *et al.*, 2001; Porwollik *et al.*, 2003), including Nm (Grifantini *et al.* 2004).

Transcriptional regulators

Four probable transcriptional regulators were upregulated

in response to H₂O₂ treatment. Fur has been previously discussed above, NGO2115 belongs to the AraC family of positive transcriptional regulators, NGO1427 shows similarity to the *cl* repressor protein of phage λ , and NGO637 belongs to a family of predicted transcriptional regulators. Additionally, three of the downregulated genes encode proteins involved in the regulation of transcription in other bacteria: *lrp* (NGO1294), *merR* (NGO602) and *hydH* (NGO1867). The *hydH* gene of *E. coli* encodes the sensor protein of a two-component regulatory system that responds to lead and zinc (Leonhartsberger *et al.*, 2001). The *merR* gene shows similarity to the MerR family of proteins, members of which co-ordinate responses to a variety of stimuli, including oxidative stress and heavy metals (Brown *et al.*, 2003), and the *lrp* gene encodes a global regulator that modulates the transition to stationary phase and regulates the transcription of over 400 genes including many involved in the response to nutrient limitation and osmotic stress in *E. coli* (Tani *et al.* 2002). Apart from Fur, none of these transcriptional regulators have been studied in Gc, so the contribution of each of these regulators to the H₂O₂ response is presently unknown.

Genes with unknown function

Approximately one-quarter of the genes upregulated in the microarray analysis (20 of 75), including two of the three most highly upregulated genes, were annotated as encoding proteins with no known functions. Over one-third (7/20) of these hypothetical proteins contained N-terminal signal sequences, suggesting that they could be secreted from the cytoplasm (NGO554, NGO1686, NGO1947, NGO1428, NGO555, NGO1948 and NGO1900). Many of the genes with proven antioxidant functions in Gc are localized to the membrane or periplasm [*msrAB* (Skaar *et al.*, 2002a), *ccp* (Turner *et al.*, 2003; Seib *et al.*, 2004), *mntC* (Tseng *et al.*, 2001) and *sco* (Seib *et al.*, 2003)], further supporting the hypothesis that these hypothetical proteins could be functioning as antioxidants.

Quantitative real-time polymerase chain reaction (Q-PCR) validates microarray data and reveals *kat* regulation

To validate our results we performed quantitative real-time polymerase chain reactions (Q-PCR) on a subset of the genes identified in our microarray analysis, measuring the expression levels of *msrAB*, *recN*, NGO554 and NGO1686, as well as the *omp3* gene, whose expression was unaffected by H₂O₂ treatment in the microarray (data not shown), in H₂O₂-treated cells and untreated controls. Increases in expression determined by microarray and Q-PCR are as follows: *msrAB* increased 3.7-fold by microarray and 2.5-fold by Q-PCR [standard error (SE) = 0.75];

recN increased 3.0-fold by microarray and 2.3-fold by Q-PCR (SE = 0.30); NGO554 increased 70.6-fold by microarray and 36.9-fold by Q-PCR (SE = 19.5); NGO1686 increased 19.3-fold by microarray and 17.15-fold by Q-PCR (SE = 8.8). Overall, a consistent differential expression was found using the two methodologies. We saw no difference in *omp3* message levels in response to H₂O₂ treatment by Q-PCR (data not shown). Earlier studies on Gc have shown that the expression of catalase enzyme is upregulated by treatment with H₂O₂ (Zheng *et al.*, 1992); however, we did not detect the *kat* transcript in our microarray analysis, which was surprising given the high level of catalase activity in Gc (Hassett *et al.*, 1990). We therefore measured the levels of *kat* mRNA in H₂O₂-treated cells and untreated controls by Q-PCR. A 51.6-fold (SE = 42.28) upregulation of the *kat* gene was observed in H₂O₂-treated cells.

Highly upregulated genes of unknown function and *recN* have roles in protection against oxidative damage

Based on the unpublished annotation initially used in conjunction with our microarray analysis (J.K. Davies *et al.*, unpubl. data), two of the genes most highly upregulated in response to H₂O₂, NGO554 (70-fold upregulation) and NGO1686 (19-fold upregulation), were predicted to encode proteins with no known function. Further analysis of these proteins using information contained in the STDGEN database (<http://www.stdgen.lanl.gov/>), including COGS and BLAST (Altschul *et al.*, 1997) analyses, as well as annotation provided by David Dyer at the University of Oklahoma (<http://www.genome.ou.edu/gono.html>), revealed that both of these proteins are predicted to contain cleavable signal sequences, suggesting that they may be secreted from the cytoplasm, and that both of these proteins show limited sequence similarity to proteins of known function.

The carboxy-terminal half of the predicted 46 kDa NGO1686 protein showed sequence similarity to the M23B family of zinc metalloendopeptidases as classified by the MEROPS protease database (<http://merops.sanger.ac.uk>). Certain members of this family, such as lysostaphin, have been demonstrated to cleave bacterial cell wall peptidoglycans, but very few members of this family have been functionally characterized (Rawlings *et al.*, 2004). The M23B family of proteases contains a HxH motif (residues 373–375 of NGO1686), where the first histidine is the active site and the second histidine binds the zinc ligand. An additional HxxxD motif found in this family (residues 295–299 of NGO1686) is also believed to bind zinc. BLAST searches using default settings revealed that the NGO1686 protein showed the highest degree of sequence similarity to uncharacterized probable peptidases from *Chromobacterium violaceum*

(gb AE002098.2; 1e-75) and *Nitrosomonas europaea* (emb BX321861.1; 2e-47). NGO1686 also showed sequence similarity to the partially characterized *E. coli* NlpD (SWISSPROT P33648) (Ichikawa *et al.*, 1994; Lange and Hengge-Aronis, 1994) and *Corynebacterium glutamicum* MepA (Möker *et al.*, 2004) proteins, both of which showed 35% sequence identity and approximately 50% sequence similarity to the carboxy-terminal third of the NGO1686 protein. However, the fact that the NGO1686 protein showed sequence similarity to small regions of MepA and NlpD suggests that the NGO1686 protein is not a functional homologue of these proteins, but is simply a zinc metalloprotease.

The most highly upregulated gene (70-fold upregulation) encoded the NGO554 protein, a Gc-specific predicted 33 kDa protein that showed no similarity to any proteins in the database, including no meningococcal proteins from the three sequenced genomes. Therefore, we wondered whether the NGO554 protein could be a virulence factor that was unique to Gc. Despite extensive sequence analysis, the only structural feature identified in this protein was a predicted cleavable signal sequence, suggesting that the NGO554 protein could be secreted from the cytoplasm. As the roles of these proteins in the response to oxidative damage, or their potential functions as antioxidants, were not patently obvious, we decided to investigate their functions in protection against oxidative damage.

We insertionally inactivated these two genes by deleting an internal portion of the gene and inserting an antibiotic resistance cassette in its place (see *Experimental procedures*), creating strains FA1090 Δ NGO1686 (Δ 1686) and FA1090 Δ NGO554 (Δ 554). Neither mutant exhibited a growth defect in liquid; however, the Δ 1686 mutant exhibited altered colony morphology when cultivated on solid agar medium, which was not a result of altered pilus or Opa expression (data not shown). We then tested the sensitivity of the mutants to the oxidative damaging agents H₂O₂, cumene hydroperoxide (an organic peroxide), paraquat (which generates intracellular superoxide) and diamide (which damages thiol groups), relative to the parent strain FA1090. The Δ 1686 mutant showed a statistically significant 30- to 300-fold increase in sensitivity to H₂O₂ at the 10, 20 and 50 mM doses (Fig. 1). The Δ 1686 mutant also showed a significant fourfold increase in sensitivity to cumene hydroperoxide at the 0.01% dose (data not shown), but showed no increase in sensitivity to either paraquat or diamide (data not shown), suggesting that the NGO1686 gene product specifically protects against oxidative damage caused by peroxides. In contrast, the Δ 554 mutant showed over a 100-fold statistically significant increase in sensitivity to the highest dose (50 mM) of H₂O₂ (Fig. 1), and showed no increase in sensitivity to any of the other oxidative damaging agents tested (data not

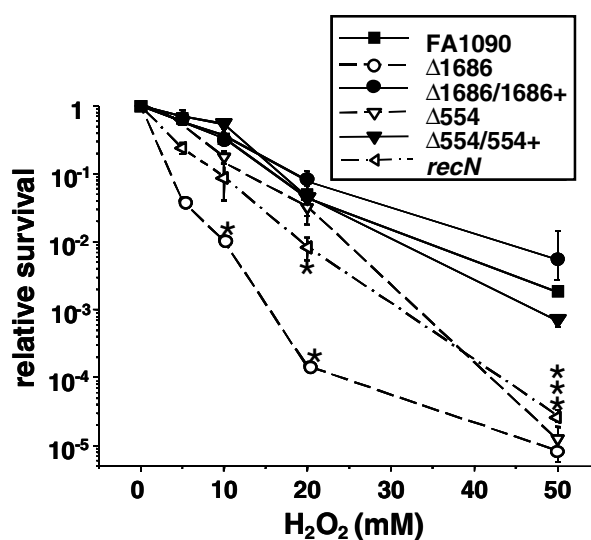


Fig. 1. H₂O₂ resistance of strains with mutations in genes upregulated by H₂O₂ treatment. Cells were treated with varying doses of H₂O₂ for 15 min and serially diluted into media containing catalase. The relative survival at each dose was calculated as the viable cfu divided by the total cfu (receiving no H₂O₂ treatment). Error bars represent the standard error of the mean of three to nine experiments for strains FA1090, Δ 1686, Δ 1686/1686+, Δ 554, Δ 554/554+ and *recN*. **P* < 0.05 by the Student's *t*-test relative to parent strain FA1090.

shown). Oxidative damage assays using a previously characterized *recN* mutant (Skaar *et al.*, 2002b) revealed that the *recN* mutant was significantly increased fivefold to the 20 mM dose and 60-fold to the 50 mM dose of H₂O₂ (Fig. 1), but showed no increase in sensitivity to the other tested oxidative damaging agents (data not shown). To ensure that the observed differences in sensitivity to oxidative damaging agents were not due to polar effects on downstream genes, we introduced functional copies of the NGO1686 and NGO554 genes at unlinked chromosomal loci either under control of the gene's exogenous promoter (NGO1686), or under control of *lac* regulatory sequences (NGO554) (see *Experimental procedures*), creating strains Δ 1686/1686+ and Δ 554/554+. In both cases we were able to complement the observed increases in sensitivity by supplying a functional copy of the gene ectopically, as strains Δ 1686/1686+ and Δ 554/554+ were statistically indistinguishable from the parental strain FA1090 and statistically different from the mutant strain in both H₂O₂ sensitivity (Fig. 1) and cumene hydroperoxide sensitivity (data not shown). These results confirmed that the phenotypes of the mutants were directly due to inactivation of the NGO1686 and NGO554 genes. We were also able to restore normal colony morphology to the Δ 1686 mutant strain by supplying a functional copy of the gene ectopically (data not shown). The altered colony morphology of the Δ 1686 mutant could be indicative of the NGO1686 protein playing a role in cell wall peptidoglycan

metabolism, as has been suggested to be the case with its closest characterized homologues (Ichikawa *et al.*, 1994; Lange and Hengge-Aronis, 1994; Möker *et al.*, 2004). However, as the $\Delta 1686$ mutant showed no alteration in MIC (minimum inhibitory concentration) to a panel of antibiotics with a variety of cellular targets (data not shown), as well as no decreased resistance to the oxidative damaging agents paraquat and diamide (data not shown), it is unlikely that the phenotype of increased sensitivity is due to increased membrane permeability but instead suggests a more specific role for the NGO1686 protein in oxidative damage protection.

recN and $\Delta 1686$ mutants show reduced survival in a PMN killing assay

Gonococcal infection results in the recruitment of large numbers of PMNs to the site of infection. PMNs perpetrate a bactericidal respiratory burst, generating substantial amounts of ROS, and also possess a variety of non-oxidative killing mechanisms. The fact that viable Gc can be found associated with PMNs suggests that Gc can survive the interaction with PMNs. To assess the contributions of *recN*, NGO1686 and NGO554 to survival in a biologically relevant model system, we measured the abilities of strain FA1090 and the corresponding mutants to survive following exposure to PMNs. Freshly isolated adherent human PMNs were co-incubated with Gc strain FA1090 or one of the corresponding mutants at a temperature permissive for bacterial adherence but not internalization. The PMN–bacteria mixture was then warmed to 37°C, and at various times thereafter the number of viable, cell-associated colony-forming unit (cfu) was assessed for each strain. Both the *recN* and $\Delta 1686$ mutants, but not the $\Delta 554$ mutant, showed a statistically significant, approximately twofold decrease in survival after exposure to PMNs relative to the parent strain FA1090 (Fig. 2 and data not shown). The $\Delta 1686/1686+$ strain complemented the mutation and was statistically indistinguishable from the FA1090 parent strain (data not shown). Taken together, these data suggest that both *recN* and NGO1686 are important for the ability of Gc to survive PMN-mediated killing and can thus be considered potential virulence factors.

Discussion

The data presented in this study comprise the first examination of the global effects of H₂O₂ on gene expression in the strict human pathogen Gc. In addition to revealing the upregulation of several genes whose products have been previously shown to be involved in resistance to oxidative stress, we have identified many novel genes with robust responses to H₂O₂ treatment. Further characteriza-

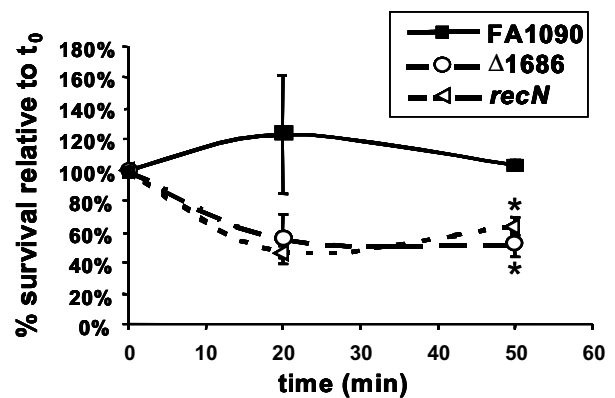


Fig. 2. Susceptibility of strains to PMN-mediated killing. Adherent, IL-8-primed PMNs were synchronously infected with Gc strains at a multiplicity of infection of 1. At various times after infection, PMNs were washed and lysed in 1% saponin before serial dilution. The number of viable cfu at each time point was expressed as the per cent of cfu enumerated at time 0 (after 10°C centrifugation). Error bars represent the standard error of the mean of at least three replicate wells, and experiments were repeated three times. * $P < 0.02$ by the Student's *t*-test relative to parent strain FA1090.

tion of the genes responding strongly to H₂O₂ demonstrated that NGO1686, a predicted zinc metalloprotease, protects against damage by two types of peroxides, and that NGO554, a Gc-specific protein, acts to protect against damage caused by high levels of H₂O₂. Our current study has also ascribed a role in oxidative damage protection to *recN*, a Fur-regulated gene (Sebastian *et al.*, 2002) involved in DNA repair in Gc (Skaar *et al.*, 2002b). A PMN survival assay allowed us to test these defined mutants in a biologically relevant system, which has revealed the importance of both *recN* and NGO1686 in resistance to PMN-mediated killing. Finally, our analysis has further underscored the link between iron, Fur and oxidative stress resistance in bacteria, specifically in the pathogenic *Neisseria*.

Although we observed that the expression of seven transcriptional regulators was altered by H₂O₂ treatment, the only one of these regulators that has been studied in detail in Gc is Fur. In some bacteria, Fur-like proteins have been shown to directly regulate the response to oxidative stress (Mongkolsuk and Helmann, 2002; Boylan *et al.*, 2003). In *E. coli* and *Bacillus subtilis*, *fur* expression increases after H₂O₂ treatment (Zheng *et al.*, 1999; Mostertz *et al.*, 2004), and in *E. coli* (Zheng *et al.*, 2001), *B. subtilis* (Mostertz *et al.*, 2004), *Pseudomonas aeruginosa* (Palma *et al.*, 2004), *N. meningitidis* (Grifantini *et al.*, 2004) and *S. typhimurium* (Porwollik *et al.*, 2003) various members of the Fur regulon were derepressed after H₂O₂ treatment. One explanation for this observation is that H₂O₂ directly inactivates the Fur protein, derepressing the Fur regulon. The resulting increases in intracellular Fur levels could lead to Fe²⁺ sequestration by Fur, thus limiting

the participation of Fe^{2+} in Fenton biochemistry ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^*$), which results in generation of the highly damaging HO^* . This Fur-dependent Fe^{2+} sequestration would be advantageous for the bacterium after exposure to H_2O_2 , and has been suggested to occur in *E. coli* (Zheng *et al.*, 1999).

In our current study, 15 iron-related or Fur-regulated genes were upregulated in response to H_2O_2 in Gc. Ducey *et al.* (2005) recently published a microarray analysis of the response of Gc strain FA1090 to iron which has substantially expanded the list of iron-regulated genes in Gc. Although a chemically defined medium was used for their analysis, while our present study was conducted in rich medium, there is an overlap in the genes regulated by iron and those upregulated by H_2O_2 treatment. Of the 75 peroxide-upregulated genes, 24 were shown to be regulated by iron: 18 were repressed by iron (*ald*, *grxB*, *fetA*, *tbp1*, *tbp2*, *pigA*, *fbpA*, *fetB*, *recN*, *exbB*, *aldA*, NGO2115, NGO2065, NGO554, NGO108, NGO322, NGO1690 and NGO1174) and six were activated by iron (NGO376, NGO775, NGO1901, NGO1686, NGO1948 and NGO865) (Ducey *et al.*, 2005). However, only 13 of these 24 genes contain a putative Fur box, as denoted by Ducey *et al.* (2005), suggesting that the mechanism of this portion of the peroxide response is not simply due to activation or repression by Fur. This finding adds to the number of bacterial species where an overlap exists between the iron and H_2O_2 response regulons. In none of these bacteria has the mechanism of this overlap been elucidated, but our data suggest that it is not simply due to Fur.

Studies of the closely related Gc and Nm have revealed that, although their genomes are very similar, they differ greatly in their pathogenicities. In contrast to what we observed in Gc, treatment of Nm with H_2O_2 only results in upregulation of 10 genes. In Nm, Fur has recently been shown to regulate expression of an operon involved in resistance to oxidative damage (NMB1436-8) (Grifantini *et al.*, 2003, 2004), and this operon is also present in Gc (data not shown). In an NMB1436-8 mutant a larger number of genes were upregulated in response to H_2O_2 , but only 20 of those genes were found in the Gc peroxide-upregulated data set (Grifantini *et al.*, 2004). Although it is difficult to directly compare these results with ours, as we treated Gc with 5 mM H_2O_2 for 15 min, whereas Grifantini *et al.* treated Nm with 200 μM H_2O_2 for 30 min, these data suggest that Gc and Nm have different transcriptional responses to oxidative damage. Further characterization of the response of both Gc and Nm to different types of oxidative damage could begin to elucidate the similarities and differences in the oxidative damage responses of these closely related but pathogenically distinct bacteria.

Of the 75 genes upregulated in our microarray analysis, we chose to study three in more detail for their resistance

to oxidative damaging agents and PMN-mediated killing. Both the *recN* and $\Delta 1686$ mutants showed increased sensitivity to H_2O_2 and killing by PMNs, whereas the $\Delta 554$ mutant only showed decreased resistance to H_2O_2 . RecN plays a role in DNA repair in both *E. coli* (Picksley *et al.* 1984) and Gc (Skaar *et al.*, 2002b) and additionally has a role in competence in *B. subtilis* (Kidane and Graumann, 2005). Although RecN has SMC domains (Skaar *et al.*, 2002b) and is an ATP-dependent single-stranded DNA (ssDNA)-binding protein (Kidane and Graumann, 2005), the exact biochemical role of RecN is unknown in any bacterium. RecN is known to be important for the repair of DNA double-strand breaks in both *E. coli* and *B. subtilis* (Picksley *et al.* 1984; Imlay and Linn, 1987; Kidane *et al.*, 2004), and a Gc *recN* mutant is extremely sensitive to DNA double-strand breaks caused by nalidixic acid (Skaar *et al.*, 2002b). *recN* is upregulated in response to H_2O_2 treatment in *E. coli* (Zheng *et al.*, 2001) and *S. typhimurium* (Porwollik *et al.*, 2003), and although it has been suggested that this is the result of induction of the SOS response by H_2O_2 -damaged DNA, this cannot be the case in Gc, which lacks an SOS response (Black *et al.*, 1998). As RecN is likely to act in the repair of oxidatively damaged DNA, it is interesting that *recN* is the only DNA repair gene upregulated in the microarray and may suggest a specific role for RecN in oxidative damage repair.

The other H_2O_2 -regulated gene shown to have a role in protection against oxidative damage and PMN killing is NGO1686. The NGO1686 protein belongs to the M23B family of zinc metalloendopeptidases, is likely to be exported from the cytoplasm and provides protection against PMN killing, as well as oxidative damage by two types of peroxides. Some members of the M23B family function to cleave peptidoglycan, and limited phenotypes have been described for other members of this family which are consistent with a role in cell wall maintenance, but none of the members of this family of proteins have been tested for a role in protection against oxidative damage. Furthermore, as no functions have been ascribed to the proteins in the database most closely related to NGO1686, we cannot use these sequence homologues as a means to predict the mechanism by which NGO1686 protects against oxidative damage.

Although the specific mechanisms by which the RecN and NGO1686 proteins protect against oxidative damage are unknown, we have clearly shown that both genes are upregulated in response to H_2O_2 and that both are important for survival to H_2O_2 and PMNs. Importantly, this is the first demonstration of gonococcal genes that affect PMN survival in the literature. The most straightforward hypothesis supported by our present data is that Gc strain FA1090 is protected from the oxidative burst of PMNs by a variety of antioxidant proteins, among them being NGO1686 and RecN, and that their removal from the

antioxidant arsenal results in increased sensitivity to PMN-mediated killing. However, an alternate hypothesis is that these proteins have differing functions in their interactions with pure H₂O₂ and PMNs, perhaps conferring survival to the non-oxidative killing mechanisms of PMNs, such as antimicrobial peptides, with H₂O₂ serving as a signal for their up-regulation in their interaction with PMNs. Intriguingly, this could suggest potential roles for the Gc- and *Neisseria*-specific hypothetical proteins found in our analysis in the complex interaction between these strict human pathogens and the human innate immune response.

Experimental procedures

Bacterial strains, growth conditions and chemicals

Escherichia coli One Shot TOP10 cells (Invitrogen) were grown on Luria–Bertani (LB) broth or agar (Difco) at 37°C to propagate plasmids. Gc strains were grown at 37°C on Gc medium base (GCB; Difco) plus Kellogg supplement I (22.2 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase) and II [1.23 mM Fe(NO₃)₃] (Kellogg *et al.*, 1963) at 37°C in 5% CO₂ or in Gc liquid (GCBL) medium [1.5% proteose peptone No. 3 (Difco), 0.4% K₂HPO₄, 0.1% KH₂PO₄, 0.1% NaCl] with Kellogg supplements I, II and 0.042% sodium bicarbonate. Unless explicitly stated otherwise, GCB and GCBL always contained Kellogg supplements I and II. Unless otherwise indicated, liquid-grown Gc strains were prepared as follows: Gc was grown from freezer stocks for approximately 20 h and ~10 colonies were passaged onto GCB. After 12 h, colonies were collected with a Dacron swab (Puritan), resuspended in GCBL at OD₅₅₀ ≅ 0.1, grown for 12 h, diluted to OD₅₅₀ ≅ 0.3, grown for 2.5–3 h, and diluted to OD₅₅₀ ≅ 0.06. This culture was grown in a flask on a shaking incubator to mid-log phase (OD₆₀₀ ≅ 0.5). All strains used in this study showed similar growth rates. All FA1090 strains used in this study contained the 1-81-S2 variant *pilE* sequence (Seifert *et al.*, 1994). Antibiotics (Sigma) were used at the following concentrations for *E. coli*: kanamycin (Kan) 40 µg ml⁻¹; erythromycin (Erm), 275 µg ml⁻¹; chloramphenicol (Cam), 25 µg ml⁻¹. For Gc, the concentrations were: Cam, 0.75 µg ml⁻¹, Erm, 0.75 µg ml⁻¹, Kan, 40 µg ml⁻¹. Antibiotic MICs were determined using *E*-test strips (AB BIO-DISK). All chemicals were obtained from Sigma unless otherwise indicated.

DNA manipulations and analysis

Standard procedures were performed as described (Sambrook *et al.*, 1989). Plasmid DNA from *E. coli*, genomic DNA from Gc, and PCR products were isolated using Qiagen kits. All modifying and restriction enzymes were obtained from New England Biolabs, unless otherwise indicated, and used as specified. For Southern blot analysis, DNA was transferred to Magnagraph nylon membranes (Micron Separations) and hybridized with Dig-labelled probes generated and used as specified by the manufacturer (Roche). Gonococcal *pilE* sequences were determined as described (Stohl and Seifert, 2001), except for sequencing reactions as below. Sequencing

reactions were performed using CEQ Dye Terminator Cycle Sequencing Quick Start Kit and CEQ 2000XL automated sequencer (Beckman Coulter) according to the manufacturer's instructions. DNA analysis was performed using VectorNTI software (Informax).

H₂O₂ treatment of Gc, RNA isolation, cDNA synthesis and fluorescence labelling

Neisseria gonorrhoeae strain FA1090 was grown as above to mid-log phase and diluted 1:10 into GCBL. The culture was split into two flasks, and half of the culture was treated with 5 mM H₂O₂ for 15 min (with shaking for both flasks) after which both cultures were treated with a final concentration of 10 µg ml⁻¹ catalase for 1 min to degrade the H₂O₂. Cultures were quickly cooled in a dry ice-ethanol bath, harvested by centrifugation for 15 min (4000 g), and pellets were stored at -80°C. RNA was isolated using the RNeasy Miniprep kit (Qiagen) with the additional on-column DNase digestion step, and quality and quantity of the RNA were assessed by agarose gel electrophoresis, spectrophotometry and Bioanalyser (Agilent). cDNA was synthesized as follows: 20 µg of total RNA of each sample was mixed with 30 µg of random hexamers (Roche), heated to 70°C for 10 min and cooled on ice for 10 min. Half microlitre of RNasin (Promega), 6 µl of Superscript II buffer (Life Technologies), 3 µl of dithiothreitol (DTT), a final concentration of: 0.50 mM for dATP, dCTP and dGTP; 0.15 mM for dTTP (Invitrogen); and 0.30 mM for aminoallyl-dUTP (Sigma), and 2 µl of Superscript II reverse transcriptase (Invitrogen) was added and incubated at 42°C for 2.5 h. Reactions were terminated and the cDNA labelled as recommended by Molecular Probes as follows: reactions were heated to 95°C for 5 min and immediately placed on ice. Aliquots of 0.42 volumes of 1 M NaOH were added and incubated at 65°C for 15 min. Solution was neutralized by addition of an equal volume (equal to amount of NaOH added) 1 M HCl and 0.11 volume of 1 M Tris-HCl (pH 7.0). Amine-modified cDNA was purified using the Qiaquick PCR purification kit according to the manufacturer's instructions, except two additional wash steps were performed, followed by ethanol precipitation of cDNA with added glycogen (Roche). The cDNAs were indirectly labelled by coupling to either AlexaFluor dye 555 or 647 (Molecular Probes) as follows: cDNA was dissolved in 5 µl of DNA, 3 µl of sodium bicarbonate (25 mg ml⁻¹) and the Alexa dye dissolved in 2 µl of DMSO (Sigma) was added to the mixture and incubated at room temperature for 1 h in the dark. The reaction was purified using the Qiaquick PCR purification kit and precipitated as above. Labelled pellets were resuspended in 4 µl of water and used within 1 h.

Microarray hybridization and data analysis

We used a pan-*Neisseria* microarray for our analysis, which will be described in detail elsewhere (J.K. Davies *et al.*, unpublished). Briefly, the microarray contains 2704 PCR products corresponding to the most conserved regions of the 6294 potential coding sequences from Gc strains FA1090 and MS11, Nm strains MC58 and Z2491, and a variety of control DNA spots, all of which are spotted in triplicate. The

printed microarray contains 99.6% of all the FA1090 annotated features. Microarrays were incubated with 32 μ l of pre-hybridization solution [(25% formamide, 5 \times SSC, 0.1% SDS, 10 mg ml⁻¹ BSA (Sigma), 1 mg ml⁻¹ salmon sperm DNA (Eppendorf)] for 45 min at 42°C inside a Corning Hybridization Chamber containing a small amount of 5 \times SSC to maintain constant moisture. Slides were washed in water and dried by centrifugation (5 min, 2000 *g*). For hybridization of the microarray slides, 4 μ l of each differentially labelled Alexafluor 555 and 647 probe was added to 24 μ l of hybridization solution (25% formamide, 5 \times SSC, 0.1% SDS, 1 mg ml⁻¹ salmon sperm DNA), the mixture was heated to 95°C for 5 min, applied to the microarray surface, and a coverslip (Corning) was added, and allowed to hybridize for 16 h at 42°C as above. Slides were sequentially washed as follows in Coplin jars: slides were placed in 2 \times SSC, 0.1% SDS until the coverslip was detached, and subsequently incubated at 42°C for 5 min in fresh solution. Slides were washed for 10 min at room temperature in 0.1 \times SSC, 0.1% SDS, 4 \times 1 min in 0.1 \times SSC, 10 s in 0.01 \times SSC, and dried by centrifugation. Slides were scanned and signal intensities were quantified using a Packard ScanArray 4000XL laser scanner and ScanArray Express version 2.1.8 software. Spots were analysed by adaptive circle quantification, local background was subtracted for each spot, and Lowess normalization was applied to calculate the log₂ Alexa647/Alexa555 median fluorescence ratios. Results were exported to Excel and statistical analyses were performed.

Q-PCR

cDNA was synthesized and purified using RNA from two biological replicates as described above except that a final concentration of 0.5 mM was used for each dNTP. PCR products to be used as standards in Q-PCR reactions were generated using the following pairs MSRAB-Q1 (5'-TGA CCGAAGAGCAATACC-3'), MSRAB-Q2 (5'-GAGCCGGAAT CATATTTGTC-3'); REC N-Q1 (5'-CTTTGGATGCGATTGGT CTG-3'), REC N-Q2 (5'-GCGGCGGATACTGAGTTC-3'); NG O1686-Q1 (5'-GGTGTGCGGCGGCATACATTG-3'), NGO1686-Q2 (5'-GCCTCCTGCACCCAATATG-3'); NGO 554-Q1 (5'-ATTTCTACCCTTTGGTTTCG-3'), NGO554-Q2 (5'-CCG TTTACATTGGTGTCTG-3'); OMP3FOR (5'-AGCAGGCT CTTCAATATGTT-3'), OMP3REV (5'-CTTGAGTCATTTGCGC TTGA-3') (Sebastian *et al.*, 2002; Sechman *et al.*, 2005); KAT-Q1 (5'-TCGCCCG TTTCACCACCGTG-3'), KAT-Q2 (5'-TTTGTGGCGGAA CGCATATTG-3'). PCR products were subsequently gel-purified (Qiaquick), and serial dilutions were included in Q-PCR runs to generate standard curves for quantification of transcripts. Q-PCR was performed on a LightCycler instrument (Roche). All assays contained 2 μ l of LightCycler DNA master SYBR Green mix (Roche), 1.6 μ l of MgCl₂ (for a final concentration of 3 mM), 2 μ l of each primer (0.5 mM final concentration), 2 μ l of template, and PCR-grade sterile water for a final volume of 20 μ l. The annealing temperature was 52°C for NGO1686, NGO554, *recN* and *msrAB*; 55°C for *omp3*; and 58°C for *kat*. Fluorescence in channels F1 and F2 was acquired at the end of every extension step, and the F2/F1 ratio was analysed. All Q-PCR assays were performed at least twice using cDNA from two biological replicates.

Construction of mutant strains

To individually mutate the NGO554 and NGO1686 genes, internal fragments of the genes were deleted and antibiotic resistance cassettes (Kan^R or Erm^R respectively) were inserted in their place. We used primer pair 554UPFWD (5'-GGCGCGCCACGTACCGCTTGAAACATATGG-3') and 554UPREV (5'-GCTAGCGTAGGAAATCGGATTCGGCAGC-3'), which introduces an NheI site (underlined), and primer pair 554DOWNFWD (5'-TTAATTAAGGCCAATCTACGGCAATG G-3') and 554DOWNREV (5'-GAGCTCGACAGGTAGTTT TCCACGATGG-3'), which introduces a PaeI site (underlined), to PCR-amplify fragments containing the 5' and 3' ends of the gene, respectively, using TaqPlusLong polymerase (Stratagene). PCR products were gel-purified and ligated to pCR2.1-TOPO (Invitrogen) to create pNGO554UP and pNGO554DOWN respectively. The kanamycin resistance cassette from EZ::TN <Kan-2> (Epicentre Technologies) was amplified with *Pfu* polymerase using primer pair KAN-2FWD (5'-GCTAGCCGCTGAACTCAAATCTCTGAT GTTACATTGC-3'), which introduces an NheI site (underlined) and a gonococcal uptake sequence (in bold), and KAN-2REV (5'-TTAATTAAGTTGATGAGAGCTTTGTTGTAGG-3'), which introduces a PaeI site (underlined). The PCR product was gel-purified and ligated into pCR-Blunt (Invitrogen) to create pBluntGUSKan-2. To clone the three fragments together, the Kan-2 cassette was excised from pBluntGUS-Kan-2 by NheI/SacI digestion and ligated to pNGO554UP digested with the same enzymes to yield the plasmid pNGO554UPKan-2. This construct was subsequently digested with SacI/PaeI and ligated to a fragment from pNGO554DOWN containing the 3' end of the gene to yield pNGO554Kan-2.

We used primer pair NGO1686-1 (5'-GCGCTGTCCCAAT TCAACACC-3') and NGO1686-2Pme (5'-CATGTTTAAAC CGGTTACCGTGTCGCAATCGG-3'), which introduces a PmeI site (underlined) and primer pair NGO1686-4 (5'-CCACAAATAACGTGCGTAAATGCCG-3') NGO1686-3Pme (5'-ATCGTTTAAACGTCAAGGTCGAAGCGG-3'), which introduces a PmeI site (underlined) to PCR-amplify fragments containing the 3' and 5' ends of the gene, respectively, using *Pfu* polymerase. PCR products were gel-purified and ligated to PCR-Blunt (Invitrogen) to create pBlunt1686DOWN and pBlunt1686UP respectively. To clone the two fragments together, pBlunt1686DOWN was digested with PmeI/BamHI, treated with Klenow and CIP, pBlunt1686UP was digested with PmeI/EcoRV to liberate the insert, and the two fragments were ligated together to yield pBlunt1686. This plasmid was digested with PmeI, treated with CIP, and a non-polar *erm* resistance cassette prepared as described (Stohl and Seifert, 2001) was cloned into the site to yield pBlunt1686Erm. All mutant constructs were sequenced to verify that only the desired mutations had been introduced. Each mutant allele was recombined into the Gc chromosome by spot transformation as described (Stein *et al.*, 1988; Stohl and Seifert, 2001) and verified by Southern blotting and PCR analysis.

To create strains to complement the mutations, we used the NICS system (Mehr and Seifert, 1998; Mehr *et al.*, 2000) to insert a functional copy of the gene ectopically at an unlinked chromosomal locus between the *lctP* and *aspC* genes, either under control of its endogenous promoter (constructs in plasmid pGCC5) or *lac* regulatory sequences

(constructs in plasmid pGCC4), and linked to either a Cam resistance cassette (pGCC5) or an Erm resistance cassette (pGCC4). Plasmid pGCC5/1686 was created by PCR amplification using *Pfu* polymerase of the NGO1686 coding region and its predicted promoter with primers NGO1686-Pac (5'-GTAGCTTAATTAACCGCCGTCAGCACATTTGCCTG-3'), which introduces a *PacI* site (underlined), and NGO1686-6 (5'-TTTTTCAGACGGCATTGTTTTTCGTGCGC-3'), with subsequent directional cloning into *PacI*/*PmeI*-digested pGCC5. Plasmid pGCC4/554 was created by PCR amplification with *Pfu* polymerase of the 554 coding region using primer pair PAC554FWD (5'-TTAATTAACCATCATATCTTTTCTTTAAAGG-3'), which introduces a *PacI* site (underlined), and 554REVPME (5'-GGTTAACCGAAAACACCGGCATTCC-3'), which introduces a *PmeI* site (underlined), with subsequent directional cloning into *PacI*/*PmeI*-digested pGCC4. Both complement constructs were sequenced to verify no mutations had been introduced, recombined into the appropriate Gc mutant strain with selection on appropriate antibiotics and verified by PCR and Southern analysis.

Oxidative damage assays

To prepare Gc for oxidative damage assays, all strains were grown in GCBL to mid-log phase ($OD_{600} \approx 0.5$) as described above and used immediately. For H_2O_2 sensitivity assays, cells were diluted 1:10 into GCBL and 5 ml aliquots were placed into 15 ml Falcon tubes. H_2O_2 was added to the tubes at final concentrations of 0, 5, 10, 20 or 50 mM and tubes were placed in a drum rotator for 15 min. Cultures were immediately serially diluted into GCBL containing $10 \mu\text{g ml}^{-1}$ catalase, and plated onto GCB agar. Colonies were counted after ~20 h growth and survival of each strain at the 5, 10, 20 and 50 mM dose of H_2O_2 was calculated relative to survival at the 0 mM dose. For cumene hydroperoxide and diamide sensitivity assays, cultures were also diluted 1:10 into GCBL and aliquoted into Falcon tubes incubated at 37°C. Cumene hydroperoxide was added to final concentrations of 0.005% and 0.01%, and diamide was added to final concentrations of 10 and 20 mM, cultures were incubated for 15 and 30 min, respectively, immediately diluted into GCBL, and plated. Relative survival was calculated as above. For paraquat sensitivity assays, cells were diluted to a final concentration of $\sim 5 \times 10^6 \text{ cfu ml}^{-1}$ in GCBL containing only Kellogg supplement I and 0.042% sodium bicarbonate in 15 ml Falcon tubes. An aliquot was removed to quantify starting cfu, paraquat was added to 0.1 mM final concentration, and tubes were placed in a drum rotor. After 20 and 40 min, aliquots were taken and immediately serially diluted into GCBL and plated onto GCB agar. Survival was calculated relative to the starting cfu.

Polymorphonuclear leukocyte killing assay

Heparinized blood was drawn from healthy volunteers, following a protocol approved by the Northwestern University Institutional Review Board. PMNs were isolated by dextran sedimentation followed by purification over a Ficoll-Hypaque gradient (Amersham) as previously described (Simons *et al.*, 2005). PMNs were resuspended in Dulbecco's PBS (without

Ca^{2+} and Mg^{2+}) (Mediatech) containing 0.1% dextrose (Fisher). Synchronized PMN infection was carried out using a protocol adapted from Brinkmann *et al.* (2004). PMNs were resuspended in RPMI (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco) and 10 nM human interleukin-8 (R&D Systems) at $2.5 \times 10^6 \text{ PMN ml}^{-1}$, and 0.4 ml of the suspension was allowed to adhere to plastic coverslips (Sarstedt) in sterile 24-well tissue culture-treated plates at 37°C for 30 min. Gc were added to each well at a multiplicity of infection of 1 and the plate was centrifuged at 400 *g* for 4 min at 10°C. Cells were washed once and replaced in RPMI with serum and warmed to 37°C. At various times after infection, cells were washed and lysed in 1% saponin in PBS before serial dilution and plate count. The number of viable cfu at each time point was expressed as the per cent of cfu present at the 0 min time point (after 10°C centrifugation). Data are expressed as the mean \pm SEM of at least three replicate wells, and experiments were repeated at least three times. Significance was determined at a 95% confidence interval by the two-tailed Student's *t*-test.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Genes downregulated in response to hydrogen peroxide.

This material is available as part of the online article from <http://www.blackwell-synergy.com>