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The DNA binding activity of the *Neisseria gonorrhoeae* LexA ortholog NG1427 is modulated by oxidation

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Summary

Neisseria gonorrhoeae is a human-specific organism that is not usually exposed to UV light or chemicals but is likely to encounter reactive oxygen species during infection. Exposure of *N. gonorrhoeae* to sublethal hydrogen peroxide revealed that the *ng1427* gene was up regulated 6-fold (Stohl *et al.*, 2005). *N. gonorrhoeae* was thought to lack an SOS system, although NG1427 shows amino acid sequence similarity to the SOS response regulator LexA from *E. coli.* Similar to LexA and other S24 peptidases, NG1427 undergoes autoproteolysis *in vitro*, which is facilitated by either the gonococcal or *E. coli* RecA proteins or high pH, and autoproteolysis requires the active and cleavage site residues conserved between LexA and NG1427. NG1427 controls a three gene regulon: itself; *ng1428*, a *Neisseria*-specific, putative integral membrane protein; and *recN*, a DNA repair gene known to be required for oxidative damage survival. Full NG1427 regulon de-repression requires RecA following methyl methanesulfonate or mitomycin C treatment, but is largely RecA-independent following hydrogen peroxide treatment. NG1427 binds specifically to the operator regions of the genes it controls, and DNA-binding is abolished by oxidation of the single cysteine residue encoded in NG1427. We propose that NG1427 is inactivated independently of RecA by oxidation.

Introduction

Neisseria gonorrhoeae (Gc) is a Gram negative diplococcal bacterium and the sole causative agent of the sexually transmitted infection gonorrhea. Gc is an obligate human pathogen that infects the genitourinary tract and associates with mucosal epithelial cells. In females, gonococcal cervicitis can cause pelvic inflammatory disease leading to permanent fallopian tube scarring, resulting in sterility and ectopic pregnancy (Edwards & Apicella, 2004). Symptomatic gonococcal infection leads to a purulent exudate consisting of polymorphonuclear leukocytes (PMNs) and exfoliated epithelial cells (Shafer & Rest, 1989).

PMNs can kill bacteria by the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Shafer & Rest, 1989, Roos *et al.*, 2003) generated by the NADPH oxidase enzyme and the nitric-oxide generating iNOS enzyme (Blackford *et al.*, 1994), and through the use of non-oxidative agents such as antimicrobial peptides and proteases (Segal, 2005). The PMN oxidative burst results in the generation of superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HClO), all of which are known to have antimicrobial activities (Root *et al.*, 1975, Thomas, 1979). The iNOS enzyme generates nitric oxide that interacts with $O_2^{\bullet -}$ to generate peroxynitrite ($ONOO^-$) (Radi *et al.*, 1991a). H_2O_2 can lead to lipid peroxidation (Fang, 2004) and the DNA lesion 8-oxo-guanine, leading to G-T transversions (Henle *et al.*, 1996). HClO and $ONOO^-$ can target DNA and

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intracellular proteins as well as cell-membrane components (Hayatsu *et al.*, 1971, Albrich *et al.*, 1981, Winterbourn *et al.*, 1992, Yermilov *et al.*, 1995, Radi *et al.*, 1991b, Radi et al., 1991a). Viable Gc are associated with activated PMNs in purulent exudates, suggesting that Gc is able to resist killing by PMNs (Shafer & Rest, 1989). Gc express opacity proteins (Opa) (Heckels, 1981) that can mediate attachment of bacteria to PMNs (Chen & Gotschlich, 1996). These Opa proteins can stimulate the PMN oxidative burst (Simons *et al.*, 2005), and Opa^- Gc induce less of an oxidative burst than Opa^+ Gc (Rest *et al.*, 1982). Since human volunteers infected with Opa^- Gc shed mostly Opa^+ Gc (Schmidt *et al.*, 2000), it is likely that this organism is experiencing oxidative radicals in the human host. Whereas actively growing Gc have been shown to abrogate the PMN oxidative burst (Criss & Seifert, 2008), dead Gc have been shown to potentiate the burst (Criss & Seifert, 2008) and both live and dead Gc are present during infection. These observations support the hypothesis that Gc has a transcriptional network in place that acts to resist oxidative killing in order to survive ROS challenge and persist in its human host.

While some of the individual proteins that protect Gc from oxidative damage have been characterized (Seib *et al.*, 2006), it is not known how Gc regulates the response to oxidative products. To determine how Gc responds to oxidative products, the Gc transcriptome profile of strain FA1090 was determined following H_2O_2 exposure (Stohl et al., 2005). 75 genes were up regulated following H_2O_2 treatment and 80 genes were down regulated. One of the three up regulated genes predicted to encode transcriptional regulators was *ng1427* (up regulated 6-fold after H_2O_2 treatment), which has sequence similarity to the SOS repressor LexA.

The SOS response system is a well characterized regulatory network in *E. coli* consisting of approximately 40 genes whose function is increase the cell's ability to undergo recombinational repair, excise damaged nucleotides, and to inhibit cell division until DNA damage is repaired (Kelley, 2006). LexA is a transcriptional repressor which controls the SOS regulon by binding to a consensus sequence (Wertman & Mount, 1985) that is present in the promoters of SOS genes including *recA*. The SOS response is activated when DNA damage results in an interruption of DNA replication, leading to the presence of stalled replication forks, and the existence of ssDNA gaps at stalled replication forks. *In vitro*, the RecA protein becomes activated by binding to single-stranded DNA and a nucleoside triphosphate (Craig & Roberts, 1980). Binding of the activated RecA to LexA leads to LexA autoproteolysis (Gudas & Pardee, 1975, Little *et al.*, 1980, Little *et al.*, 1981) and subsequent derepression of the SOS regulon. Genes of the *E. coli* SOS response include *uvrA*, *uvrB*, and *uvrD* (Little & Mount, 1982), *recA*, and *lexA* itself (Brent & Ptashne, 1980). The conserved LexA binding site is not present in the Gc genome, and no gene encoding a homolog to LexA has been previously described in *Neisseria* (Davidsen & Tonjum, 2006). Additionally, following treatment of Gc with DNA damage-inducing agents such as UV light or methyl methanesulfonate (MMS), the transcription of genes such as *recA*, *uvrA*, and *uvrB* are not up regulated (Black *et al.*, 1998). Interestingly, although Gc does not up regulate DNA repair genes such as r*ecA*, *uvrA*, *or uvrB* following DNA damage, the up regulation of the putative LexA ortholog *ng1427* and numerous other genes by hydrogen peroxide suggested that a SOS-like system may exist in Gc. In support of this suggestion, we find that NG1427 acts as a repressor of a three gene regulon and that NG1427 retains RecA-facilitated autoproteolysis, but that NG1427 can be inactivated independently of RecA by oxidation.

Results

NG1427 is a transcriptional repressor

A bioinformatics approach was taken to characterize the NG1427 protein in Gc Strain FA1090 (accession #AE004969). Utilizing the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/protein?db=cdd), NG1427 showed similarity to the SF clan of serine peptidases (Barrett & Rawlings, 1995), specifically the S24 peptidase family (Little *et al.*, 1994). The canonical member of the S24 peptidase family, the SOS-response repressor LexA has 22% amino acid identity and 37% amino acid similarity to the NG1427 protein (Fig. 1A) and a similar arrangement of catalytic domains (Fig. 1B). The dissimilarity in the N-terminal region where the putative DNA binding domain of NG1427 would be located was expected since the SOS binding box in not found in Gc and NG1427 would likely have a different set of amino acids to bind to a unique promoter sequence. NG1427 encodes the conserved S24 cleavage site (A112/G113) and the conserved serine/lysine catalytic dyad (S154 and K190).

To determine the function of NG1427, a Gc loss-of-function mutant was created by deleting approximately 45% of the coding sequences and inserting a chloramphenicol-resistance cassette (*cat*) in its place (FA1090 1-81-$S2_{nv}$ *ng1427::cat*). Microarray analysis was used to compare gene expression differences between *ng1427::cat* and the parent strain. In the *ng1427::cat* mutant three genes showed increased transcript levels relative to the parent strain: the *ng1427* gene itself, *ng1428*, and *recN*. No genes showed decreased transcript levels (data not shown). The *ng1427* transcript was increased 3.6-fold in *ng1427::cat* relative to the parental strain. Although the *ng1427* transcript was interrupted by the *cat* cassette, the intact 5' end of the transcript hybridized to the microarray, an observation we exploited for real-time quantitative PCR (qPCR) analysis (see below). The *ng1428* gene showed 16.5-fold higher transcripts in the mutant and encodes a *Neisseria*-specific gene, predicted to be an integral membrane protein. The NCBI Conserved Domain Database did not detect any conserved domains in *ng1428*. The genes *ng1427* and *ng1428* are adjacent and divergently transcribed (Fig. 1C). The final gene of the NG1427 regulon, *recN*, showed a 9.8-fold increase in transcripts in *ng1427::cat* relative to the parental strain. The RecN gene product has been implicated in Gc DNA repair processes (Skaar *et al.*, 2002) and contributes to Gc survival after exposure to ROS and PMNs (Stohl et al., 2005). These results show that the LexA homolog NG1427 encodes a self-repressing transcriptional repressor, but the only gene affected by NG1427 that has previously been found in an SOS regulon is *recN*.

To verify the microarray results, qPCR was performed on RNA isolated from exponentially growing *ng1427::cat* and parental Gc. qPCR analysis of basal gene expression from *ng1427::cat* and parental Gc corroborated the microarray data, confirming that NG1427 represses the three-gene regulon of *ng1427*, *ng1428*, and *recN*, hereafter referred to as the "NG1427 regulon" (Fig. 1D, white bars). Analysis of *recA* gene transcripts confirmed that *recA* is not controlled by NG1427 (Fig. 1D). These results confirm that *ng1427* encodes a self-repressing transcriptional repressor, but that the only gene controlled by NG1427 that has previously been found in an SOS regulon is *recN*. To affirm that de-repression of the NG1427 regulon is due to the absence of *ng1427*, a wild-type copy of *ng1427* was expressed under control of its native promoter in an unlinked locus in the Gc chromosome (Mehr *et al.*, 2000). In strain Δ*ng1427*/*nics2::ng1427* the repression of *ng1428* and *recN* was restored to near, but not identical to, parental levels, illustrating that the transcriptional effects we detected were due solely to the *ng1427* mutation (Fig. 1D, gray bars). *ng1427* expression was measured by qPCR in the complemented strains but due to the fact that *ng1427* was not expressed from the wild-type locus in the complemented strains these data were not included in Fig. 1. The data regarding the Δ*ng1427*/*nics2::g113d* and Δ*ng1427*/*nics2::c64s* are

discussed below. Taken together, the microarray and qPCR analysis shows that *ng1427* encodes a transcriptional repressor of a three-gene regulon.

NG1427 undergoes RecA-mediated autoproteolysis

Since NG1427 is predicted to be a S24 peptidase like LexA, which requires RecA for autoproteolysis and SOS regulon de-repression, we directly tested whether NG1427 undergoes RecA-mediated autoproteolysis. NG1427-HIS was purified as a recombinant protein from *E. coli*. Incubation of NG1427-HIS with either activated Gc or *E. coli* RecA promoted autoproteolysis (Figure 2A, lanes 5, 6, and 8). When we performed this assay under the same conditions but substituting *E. coli* LexA for NG1427-HIS, LexA was cleaved much faster than NG1427-HIS under the same conditions (data not shown). Interestingly, we observed that NG1427-HIS underwent limited autoproteolysis even in the absence of RecA (Fig 2A, lanes 1 and 2), despite purification of the protein from *E. coli* cells lacking RecA and in the presence of protease inhibitors. We also found that NG1427-HIS underwent limited autoproteolysis immediately during purification and that this autoproteolysis was pH-sensitive, a shared trait between this protein and *E. coli* LexA (Little, 1984) (data not shown). To demonstrate that, as with LexA, activated RecA facilitated the autoproteolysis of NG1427-HIS, the reaction was also performed in the absence of RecA (mock-treated with RecA storage buffer) (Figure 2A, lane 2) and in the absence of ssDNA (lane 4). Both of these conditions resulted in less proteolysis of NG1427-HIS compared to the amount of proteolysis observed in the complete reaction (lanes 5). We found that pre-treatment of NG1427-HIS with 15mM of the oxidizing agent H_2O_2 or the reducing agent DTT did not alter the ability or extent of RecA- promoted NG1427-HIS autoproteolysis when added to the cleavage reaction (data not shown). We also observed no increase in RecA-promoted NG1427-HIS autoproteolysis when we incubated with a three-fold increase of RecA and ssDNA in the cleavage reaction. These results suggest that there remains a population of NG1427-HIS that is recalcitrant to RecA-promoted autoproteolysis for reasons that remain unclear.

To determine whether the conserved S24 peptidase cleavage site of NG1427 was the site of RecA-facilitated cleavage, the residue was mutated (NG1427-G113D), and NG1427-G113D-HIS protein was purified from *E. coli*. NG1427-G113D-HIS and NG1427-HIS proteins were incubated with purified Gc or *E. coli* RecA. Incubation of NG1427-HIS with either activated Gc or *E. coli* RecA resulted in efficient autoproteolysis (Fig. 2A, lanes 6 and 8), whereas the NG1427-G113D-HIS mutant remained intact (Fig. 2A, lanes 7 and 9), verifying that Gly113 is required for proteolytic cleavage.

To determine whether NG1427 undergoes RecA-facilitated autoproteolysis in Gc, the *ng1427* mutant was complemented with a 3’ hemagglutinin epitope (HA)-tagged version of *ng1427*, expressed under control of the *ng1427* promoter. This tagged complement construct was also introduced into a *ng1427* mutant also carrying a *recA* loss-of-function allele, *recA4*, (Seifert, 1997). We monitored autoproteolysis of the NG1427-HA protein following treatment of the liquid grown Gc with oxidants, the DNA cross linking agent mitomycin C (MMC), or the DNA alkylation agent MMS. There was a slight increase in NG1427-HA breakdown product when NG1427-HA was exposed to H_2O_2, and minimal, if any, turnover of NG1427-HA turnover occurred following exposure the ROS agents $ONOO^-$, or the hypochlorous-acid-generating chemical sodium hypochlorite (NaOCl) in the presence or absence of RecA (Fig. 2B). This suggested that either these agents are not causing DNA damage *in vivo* to activate RecA, or that NG1427-HA turnover is not mediated by RecA. In contrast, RecA-mediated turnover of NG1427-HA was nearly complete following treatment of Gc with MMC or MMS (Fig. 2B). These results suggest that DNA damage by MMC and MMS is leading to activation of RecA, causing turnover of NG1427 in the bacterial cell.

The NG1427 regulon is de-repressed independently of RecA following H_2O_2 treatment

Given the lack of RecA-mediated autoproteolysis of NG1427 following H_2O_2 treatment, we tested the RecA dependence for regulon de-repression using a strain carrying the IPTG-inducible *recA* gene, *recA6* (Seifert, 1997). Gc were treated with H_2O_2 in the presence and absence of IPTG, and expression levels of the NG1427 regulon were measured relative to a reference gene, *omp3*. We observed that treatment with H_2O_2 caused de-repression of the NG1427 regulon, irrespective of RecA expression (Fig. 3A). To confirm the RecA-independent observation, NG1427 regulon de-repression following H_2O_2 treatment was determined in the *recA4* loss-of-function strain background. NG1427 regulon de-repression still occurred after H_2O_2 treatment in *recA4* Gc (Fig. 3B, dark gray bars, dark gray striped bars) at a level similar to that of the parent (Fig. 3B, white bars, white gridded bars). No increase in NG1427 regulon gene expression was observed in the *ng1427::cat* strain after H_2O_2 treatment (Fig. 3B, light gray bars, light gray gridded bars), as these genes were already fully de-repressed. These data show that NG1427 regulon de-repression in response to H_2O_2 requires NG1427, but is independent of RecA.

NG1427 requires RecA for full regulon de-repression following DNA damage

Given that RecA facilitated NG1427 autoproteolysis, we tested whether the NG1427 regulon was induced by MMS or MMC treatment and whether this required RecA. De-repression of the *ng1427* regulon was observed in the parent strain in response to MMS and MMC (Fig. 4A, white bars, white gridded bars; Fig 4B, white bars, white gridded bars). De-repression of the NG1427 regulon was not seen in the *ng1427::cat* strain (Fig. 4A,light gray bars, light gray gridded bars; Fig 4B, light gray bars, light gray gridded bars) as the regulon was fully de-repressed. A slight, but inconsistent up regulation of the NG1427 regulon that was less than the increase of transcript levels observed in the parental strain occurred in *recA4* Gc following MMS and MMC treatment, suggesting an additional minor RecA-independent pathway of NG1427 regulon regulation may exist (Fig. 4A and 4B, dark gray bars, dark gray striped bars). These results suggest that, similar to LexA in *E. coli*, treatment with these DNA damaging agents activates the RecA co-protease within Gc to promote cleavage of NG1427 and induction of the regulon. The regulon was not induced by heat shock (data not shown), suggesting that the induction of the NG1427 regulon is specific to DNA damaging agents. Additionally, the NG1427 regulon was was not greatly affected following treatment with $ONOO^-$ (Fig. 5A) and NaOCl (Fig. 5B) in either RecA+ or RecA- cells. Therefore, NG1427 responds to different DNA damaging agents by de-repression of the NG1427 three-gene regulon in a RecA-dependent manner following non-oxidative DNA damaging agent treatment, and in a RecA-independent manner following treatment with H_2O_2.

Mutation of the single cysteine of NG1427 (C64) affects DNA binding and RecA-mediated turnover of NG1427

One notable difference between NG1427 and the *E. coli* LexA protein is the presence of a single cysteine residue near the DBD domain of NG1427 (Fig. 1A, 1B). Cysteine residues can become modified by ROS to form sulfenic acid (Winterbourn & Hampton, 2008), leading to an inter- or intramolecular disulfide bond with other cysteine amino acid residues (Thornton, 1981). We postulated that the C64 residue was becoming oxidized during H_2O_2 treatment, leading to inability of NG1427 to bind DNA. To test whether disruption of C64 altered the ability of NG1427 to bind DNA, a C64S mutant of NG1427 was constructed and introduced into an ectopic site on the chromosome of *ng1427::cat* using the NICS complementation system to generate the strain Δ*ng1427/nics2::c64s*. The C64S complement construct did not restore the *ng1427::cat* transcriptional phenotype (Fig. 1D, black bars), as the NG1427 regulon remained de-repressed following expression of *c64s*. Since disruption

of residue C64 prevented complementation of *ng1427::cat*, the presence of this cysteine residue must be required for required for NG1427 repressor activity.

To test whether mutation of the C64 residue influenced RecA-enhanced autoproteolysis, an HA-tagged version of the NG1427-C64S mutant was constructed and introduced into the *ng1427* mutant by complementation. Treatment of cells with H_2O_2, $ONOO^-$, NaOCl, MMC, or MMS incurred minimal turnover of NG1427-C64S-HA relative to the untreated samples (Fig. 6), suggesting that the C64 residue is important for RecA-mediated turnover.

NG1427 binds specifically to promoter regions, and oxidatively treated NG1427 is unable to bind DNA

To directly test whether oxidation of the C64 residue would inhibit NG1427 repressor activity, we utilized an electrophoretic mobility shift assay (EMSA) to detect DNA binding of NG1427 to the *ng1427-ng1428* intergenic region and the *recN* promoter region. First, purified non-cleavable NG1427-G113D-HIS protein was incubated with the putative operator regions of *ng1427*/*ng1428* and *recN*. NG1427-G113D-HIS was utilized due to its stability under all conditions tested and because *g113d* was able to restore the repression phenotype of *ng1427::cat* to near parental levels *in vivo* (Fig. 1D, dark gray bars). Competition EMSAs demonstrated that NG1427-G113D was able to bind specifically to the putative promoter regions of the NG1427 regulon and not to DNA carrying a viral transcription factor (EBNA) target sequence (Fig. 7, lanes 9–11, 20–22). The NG1427-G113D protein bound the *ng1427/ng1428* intergenic region at higher affinity than the *recN* intergenic region (Fig. 7, lanes 14–16) as determined by diminution of bound probe and increase of free probe. This binding could be abrogated by the addition of oxidative agents to the reaction. Abrogation of DNA binding by NG1427-G113D was seen at approximately 3 mM H_2O_2 (Fig 8A) and binding activity was restored with the addition of DTT to the reaction mixture (data not shown). With $ONOO^-$, the loss of binding occurred at 0.2mM (Fig. 8C), and with NaOCl the abrogation of DNA binding occurred at 2 μM (Fig. 8D). There was no effect on DNA binding with MMS (Fig. 8B) or degraded $ONOO^-$ (data not shown). We attempted to measure the rates of oxidation mediated by H_2O_2, or $ONOO^-$, but could only determine that abrogation of DNA binding of NG1427-G113D occurred in less than 30 seconds of exposure regardless of the dose of oxidant used (data not shown).

NG1427 is directly modified by oxidative chemicals

While we were able to infer oxidation of NG1427 by abrogation of DNA binding, we wished to determine whether the protein was becoming directly oxidized by our oxidizing agents. Again, NG1427-G113D-HIS was utilized due to its stability under all conditions tested and because it was able to restore near, but not identical, parental levels of NG1427 regulon repression *in vivo* (Fig 1D, dark gray bars). Oxidation of NG1427-G113D was detected using maleimide-PEG11-biotin (Pierce) to label unmodified cysteine residues, and labeling was detected by both a mobility shift and detection of the biotin by streptavidin-HRP. There was a loss of labeling on NG1427-G113D-HIS by maleimide-PEG11-biotin following treatment with all the oxidative agents (Fig. 9A). Additionally, we observed via Coomassie-stained protein gel the formation of a higher molecular weight structure that corresponded to a NG1427-G113D dimer, most likely created by an intermolecular disulfide-bond (Fig. 9A). This higher molecular weight structure disappeared when samples were treated with the reducing agent β-mercaptoethanol (Fig. 9C). Additionally, there appeared to be a loss of binding of maleimide-PEG11-biotin following treatment with a reducing agent, suggesting less-efficient binding was occurring following reduction of the label (Fig. 9D). Lack of detection of biotin labeling in a higher molecular weight form of the protein following H_2O_2 and NaOCl treatment (Fig. 9B) showed that there were no free sulfhydryl groups available to bind the malemide-PEG11-biotin, confirming that the dimer

was due to disulfide bonding. Detection of the biotin label in the higher molecular weight form of NG1427-G113D following treatment with $ONOO^-$ despite β-mercaptoethanol treatment suggests that this higher molecular weight structure is not due to the formation of the disulfide-bond (Fig. 9C, 9D). This insensitivity of the higher mobility form of the protein to reduction was unexpected, but formation of non-reducible protein aggregates following treatment with $ONOO^-$ has been observed previously (Soszynski & Bartosz, 1996). These results demonstrate that NG1427-G113D is clearly becoming directly and reversible oxidized by H_2O_2 and NaOCl.

Discussion

We have identified a LexA ortholog in the obligate human pathogen *N. gonorrhoeae*, NG1427, which undergoes a RecA-independent de-repression of its regulon following oxidation. NG1427 was the most highly up regulated putative transcriptional regulator in Gc following treatment with H_2O_2 (Stohl et al., 2005). Analysis of the transcriptome of Gc revealed that NG1427 is a transcriptional repressor that controls a three gene regulon of itself, *ng1428* and *recN*. The small size of the NG1427 regulon is consistent with other characterized Gc regulons, which vary from 3–12 genes in size (Kidd *et al.*, 2005, Seib *et al.*, 2007, Wu *et al.*, 2006).

Although the *in vitro* data clearly shows that activated RecA can facilitate the autoproteolysis of NG1427, full de-repression of the NG1427 regulon following oxidative DNA damage does not require RecA activity. This is the first fully-characterized S24 peptidase transcriptional repressor to have a RecA-independent mode of regulation. There was a slight but inconsistent increase of NG1427 regulon expression measured in the *recA4* strain following MMS and MMC treatment suggesting a minor, oxidation- and RecA-independent pathway for NG1427 regulon activation may exist. It is interesting that other oxidatively-damaging, thiol-modifying agents such as $ONOO^-$ or NaOCl did not strongly affect the NG1427 regulon even though they are quite potent at inactivating the DNA-binding activity of NG1427 *in vitro*. Either these oxidants are not as stable as H_2O_2 in Gc, or there may be an intermediate required for NG1427 inactivation with the bacterial cell that is responsive to hydrogen peroxide but not the other oxidants.

NG1427 possesses a single cysteine, C64, near the DNA binding domain. The –SH group on Cys residues can be modified by reactive oxygen species to form sulfenic acid (Winterbourn & Hampton, 2008). Several transcriptional regulators such as OxyR, FurR and OhrR function by using Cys residues as sensors for an oxidative environment (Chen *et al.*, 2008, Katona *et al.*, 2004, Fuangthong & Helmann, 2002, Zheng *et al.*, 1998, Paget & Buttner, 2003). A reactive oxygen species-mediated modification of the -SH group on C64 to a sulfenic acid could be altering protein structure. Indeed, mutation of C64 results in an inability of this protein to restore repression of the NG1427 regulon and to undergo RecA-mediated turnover within Gc cells. Moreover, we detected direct oxidation of NG1427-G113D by the presence of free sulfhydryl groups and formation of disulfide bond- mediated dimers *in vitro.* We were unable to purify C64 mutants in order to more directly test the oxidation of the Cys residue in NG1427- disruption of this particular residue causes the protein insolubility (data not shown). This suggests that C64 is important for proper folding and structure of NG1427. The G113D complement construct was used in an attempt to demonstrate that a non-cleavable NG1427 protein could undergo RecA-independent regulon de-repression following H_2O_2 treatment. However, we had minimal induction from the NICS locus following treatment with a variety of agents using a range of complementation constructs (data not shown), yet we still observed restoration of the transcriptional repression phenotype (Fig. 1D, data not shown). This observation is intriguing and is a current focus of ongoing study. There are no cysteine residues found in the LexA proteins of

E. coli or *B. subtilis*, but a subset of bacterial species including *Pseudomonas aeruginosa*, *P. fluorescens*, *Vibrio splendidus*, and *Azotobacter vinelandii* do encode a single cysteine, suggesting these repressors may also be directly regulated by oxidation.

It is possible that NG1427 senses ROS in the presence of activated innate immune cells and that up regulation of the three gene regulon provides a survival advantage that our *in vitro* assays cannot detect. However, since the genes found in this regulon are almost entirely conserved in the sequenced genus *Neisseria* (*ng1428* is not present in, or is not significantly conserved in, *N. elongata*, *N. subflava*, *N. mucosa*, or *N. oral* taxon 014), and both pathogenic and nonpathogenic members of the species carry NG1427 orthologs with the conserved cysteine, it is likely that this regulon is important for human colonization and not pathogenesis *per se*. It should be noted that the commensal *Neisseria* probably interact with the innate immune system as they establish and maintain colonization. It is unknown whether Gc or the other *Neisseria* encounter the type of host-derived DNA damage that cause replication fork stalls that would lead to RecA activation and NG1427 autoproteolysis. Thus, it is possible that the regulation of the NG1427 regulon through oxidation is the primary mechanism for transcriptional control and that the autoproteolysis is a residual function that is retained due to structural requirements. Also, given the slight up regulation observed in the absence of RecA and non-oxidative damage, there might exist yet another mechanism for NG1427 regulon control.

Previous investigation into the Gc response to DNA-damaging agents concluded that there was no LexA-ortholog encoded by Gc (Black et al., 1998, Campbell & Yasbin, 1984). While NG1427 is a functional LexA homolog, it is not participating in a “classical” SOS system since it does not regulate *recA*. LexA represses SOS genes by binding to a consensus sequence (Wertman & Mount, 1985), conventionally referred to as SOS boxes. This SOS binding box consensus sequence can vary from species to species. In *E. coli* and *B. subtilis*, the SOS box consists of a palindromic repeat of CTGTA-N_6-TACAG and GAAC-N_4-GTTC respectively (Erill *et al.*, 2007), although there are reports of divergent SOS boxes in *Alphaproteobacteria* that are characterized by an odd-numbered spacer and a direct repeat of GTTC-N_7-GTTC (Erill et al., 2007). However, the intergenic regions upstream of *ng1427/ng1428* and *recN* do not encode any conserved LexA binding site, and analysis using MEME/MAST (http://meme.sdsc.edu/meme4_3_0/intro.html) did not reveal an alternative putative NG1427 binding site. The different levels of gene expression changes within the NG1427 regulon after induction suggests there may be variability in the strength of NG1427 binding to its operators.

It has not been clearly defined what defines a bacterial SOS system. NG1427 is a functional homologue of LexA that is auto regulatory, but it does not control *recA* expression or the expression any DNA repair gene except *recN*. In addition, NG1427 does not affect Gc’s survival to a variety of DNA damaging or redox perturbing agents in any appreciable manner under the laboratory growth conditions used, either when deleted or over expressed (Fig. S1). *recN* was previously shown to be required for Gc survival against oxidative damage and PMN-mediated killing (Stohl et al., 2005), but deletion of *ng1428* does not provide a significant phenotype compared to the parental strain of Gc following treatment with H_2O_2 (Fig. S1A). We therefore hypothesize that basal expression of *recN* is sufficient to protect Gc from oxidative damage under laboratory growth conditions and that there are redundant processes that mask Δ*ng1427* and Δ*ng1428* survival phenotypes. Based on the additional regulation of NG1427 by oxidation and the major differences between the Gc and *E. coli* regulons, we conclude that the *Neisseria* encode an SOS-like regulon.

Experimental Procedures

Bacterial strains, growth conditions, and chemicals

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 $Fe(NO_3)_3$] (Kellogg *et al.*, 1963) at 37°C in 5% CO_2 or in Gc liquid (GCBL) medium [1.5% proteose peptone No. 3 (Difco), 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl] with Kellogg supplements I, II and 0.042% sodium bicarbonate. GCBL always contained 0.042% sodium bicarbonate, except for $ONOO^-$ assays. Liquid-grown Gc strains were prepared as follows: Gc was grown from freezer stocks for approximately 20h and ~10 colonies were passaged onto GCB. After 12h, colonies were collected with a Dacron swab (Puritan), resuspended in GCBL, grown for 12h, diluted to $OD_{550}\cong0.3$, grown for 2.5–3h, and diluted to $OD_{550}\cong0.06$. This culture was then grown to an OD_{600} of approximately 0.5. All FA1090 strains used in this study contained the 1-81-S2 variant *pilE* sequence (Seifert *et al.*, 1994) and which, other than the *recA6* strain used in this paper, contain a transposon mutation upstream of the *pilE* locus that blocks antigenic variation (1-81-S2 non-varying or 1-81-$S2_{nv}$) (Sechman *et al.*, 2005). The *E. coli* plasmids pSmart (Lucigen) and pBlunt (Invitrogen) were used for cloning, and were propagated using *E.coli* TOP10 cells (Invitrogen).

DNA manipulations and analysis

Standard procedures for cloning, Southern blot analysis, and restriction digest 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 (NEB), unless otherwise indicated, and used as specified. Gonococcal *pilE* sequences were determined as described (Stohl & Seifert, 2001). Sequencing reactions were performed using a commercial vendor (Seqwright). Analysis of DNA sequences was performed using VectorNTI (Invitrogen).

Construction of the Insertion/Deletion Mutants ng1427::cat and ng1428::tetM

An insertion/deletion mutation of *ng1427* was created by amplifying the 5' and 3' ends of *ng1427* along with flanking regions using *ng1427* 5, 6, 7, and 8 primers (Table S1). *ng1427* primers 6 and 7 encoded a *Pme*I site, which allowed the insertion of a chloramphenicol (Cam) resistance cassette (*cat*). The resulting construct, *ng1427::cat*, lacks 311 bp from the interior of the 708bp *ng1427* gene. This construct was transformed into FA1090 1-81-$S2_{nv}$ and integrated into the chromosome by homologous recombination, and transformants were selected for growth on GCB containing 0.75 μg/ml Cam. PCR and Southern blot analysis was used to verify *ng1427::cat* insertion. The *ng1428::tetM* mutant was constructed by removing 200 bp internal to *ng1428* by amplifying the 5' and 3' ends of the gene, along with flanking regions, with NG1428 1, 2H, 3H, and 4 primers. Subsequent cloning into pBlunt and subsequent digest with *Xba*I and *Bam*HI allowed insertion of a tetracycline (Tet) resistance cassette TetM (Seifert, 1997), resulting in *ng1428::tetM*. The *ng1428::tetM* construct was sequenced, transformed into FA1090 1-81-$S2_{nv}$, integrated into the chromosome by homologous recombination, and screened using antibiotic selection of 0.75 μg/ml of Tet, and confirmed by PCR.

Point Mutation Generation

NG1427-G113D and NG1427-C64S were created using the Stratagene QuikChange Mutagenesis protocol. PCR was performed using the mutagenic primers with the *pBlunt*/*ng1427* construct as a template. Following completion of the PCR, the template circular DNA was digested using *Dpn*I for 90 minutes at 37°C. The remaining DNA was cloned into

TOP10 *E. coli* cells (Invitrogen) and screened on antibiotic media. Plasmids were extracted by miniprep (Qiagen) and sequenced.

Protein Purification

ng1427 was amplified with primers NG1427 Ab2.2 and NG1427 nostop2 that allowed cloning into the IPTG-inducible pET28(a) Protein Expression Plasmid (Novagen), which introduced a C-terminal HIS tag (NG1427-HIS). Plasmids were selected on Kan antibiotic, sequenced to validate the correct insertion of *ng1427*, and transformed into BLR(DE3) expression cells (Novagen).

To isolate the NG1427-HIS protein, 1 ml of overnight culture of pET28-NG1427-HIS in LB-Kan was inoculated into 500 ml of LB/Kan. The culture was grown with vigorous shaking at 37°C for 3 hours, then treated with 1 mM IPTG for another 3 hours. The culture was pelleted at 10,000g for 10 minutes at 4°C. The cell pellet was directly mixed with 2 mM PMSF and 2x Protease Cocktail Inhibitor (Pierce), resuspended in 10 ml of Binding Buffer (Novagen), sonicated on ice for 15 seconds with 60 seconds between sonications, and pelleted for 30 minutes at 10,000g at 4°C. The supernatant was passed through a 0.45 micron filter and applied to a Novagen Quick-Bind Column. Washed NG1427-HIS was eluted from the column in 10ml of Elution Buffer (Novagen). The protein eluate was diluted 1:300 in 25 mM Tris, 150 mM NaCl, 20% glycerol pH 8.3, dialyzed with a Slide-a-Lyzer (Pierce), snap-frozen in dry-ice/ethanol, and stored at −80°C. This protocol was repeated for the NG1427-G113D and point mutation, except without PMSF and protease inhibitor treatment. Purity of the protein was assayed by loading purified protein onto a 17% SDS-PAGE, subjecting to electrophoresis, and staining with Coomassie Brilliant Blue.

Complementation

Complementation of the *ng1427::cat* mutation was performed using the NICS system (Mehr et al., 2000) which inserts a functional copy of the gene at an unlinked chromosomal locus. *ng1427* was placed under endogenous promoter control (*nics2)* and placed into the plasmid pGCC2 using the IGR1427 and Comp3' primers. The IPTG-inducible *ng1427* and *g113d* complement constructs (*nics4)* were cloned into the pGCC4 plasmid using primers *Pac*I-NG1427-CF and *Fse*I-NG1427-CR. PCR products and plasmids were digested using *Pac*I and *Fse*I and purified using the Qiaquick kit (Qiagen) and subsequently ligated. After selection on 250 μg/ml Erm colonies were screened for plasmids bearing the insert by PCR. Complement constructs were sequenced to verify no additional mutations had been introduced, transformed into *ng1427::cat* Gc, selected on antibiotics, and verified by PCR.

Microarrays

Parental and *ng1427::cat* Gc were grown to mid-log phase in amended GCBL with the RNA extracted as described below. RNA quality was determined by 2100 Bioanalyzer (Agilent) by the Northwestern University Genomics Core and by spectrophotometer. Six biologically independent samples of RNA consisting of three samples from each of the Parental and *ng1427::cat* Gc were processed and analyzed by Nimblegen (Madison, WI, USA).

Quantitative PCR Primer/Probe Design

A fluorescent probe hybridization approach was used for the quantitative PCR (qPCR) analysis of gene expression in Gc. For *ng1427, ng1428,* and *recN* probes, the Beacon Design program (http://www.premierbiosoft.com/molecular_beacons/index.html) was used to determine the best primer/probe set for each gene. Each probe has a 5'-FAM fluorescent tag and a 3'-TAM quencher. For *recA, ng1427 5' end,* and *omp3,* probes, the Integrated DNA Technology Sci-Tools website (http://www.idtdna.com/scitools/scitools.aspx) was used to

determine the best primer/probe set for each gene. Each probe was diluted to a storage stock of 300 μM in DEPC-treated H_2O prior to use.

RNA extraction and cDNA synthesis

Gc were grown in liquid culture to mid-log phase (*Stohl et al. submitted*). For production of cDNA for examination of basal gene expression, 1 ml aliquots of mid-log phase Gc were taken. To examine the role of H_2O_2, $ONOO^-$, NaOCl,MMC, or MMS on Gc gene expression, 1 ml aliquots of mid-logarithmic phase Gc were diluted 1:10 in amended GCBL. An untreated culture was maintained, and the experimental culture was treated with either 5 mM H_2O_2, 3 mM $ONOO^-$ (Fisher) in GCBL lacking sodium bicarbonate, 1.7 mM NaOCl (Sigma), 25 ng/ml MMC (Sigma), or 0.02% v/v MMS (Sigma). After incubation with shaking at 37°C for 15 minutes (H_2O_2, $ONOO^-$, NaOCl), 30 minutes (MMC), or 60 minutes (MMS), 1 volume of Gc culture was added immediately to 2 volumes RNAprotect Bacteria Reagent (Qiagen). RNA extraction was performed using the RNeasy Mini kit (Qiagen), with an in-solution DNase digestion step at the end of the extraction. Purity and concentration of RNA was assayed by spectrophotometer. cDNA was synthesized as described (Stohl et al., 2005) with Superscript III (Invitrogen).

qPCR

qPCR was performed using gene-specific fluorescent probes, with samples not treated with reverse-transcriptase and a non-template sample as negative controls. Each reaction contained 12.5 μl of iQ Supermix (Bio-Rad), 0.15 μl (300 nM final conc) primer, 2.5 μl probe (final conc 0.3 μM), 4.7 μl H_2O, and 5 μl of 1/100 diluted cDNA. Reactions were run using the iQ5 Real-Time PCR Detection System (Bio-Rad). Reaction conditions were as follows: 1 cycle of 95°C for 3:00 minutes; 40 cycles of 95°C for 00:10 minutes, 58°C for 00:30 minutes. *Omp3*, a gene encoding an outer-membrane protein not regulated by ROS, was used as a control (Stohl et al., 2005). *ng1427* expression in *ng1427::cat* was measured with the “ng1427::cat 5’ end qPCR” primer/probe set that detects *ng1427* transcript upstream of the *ng1427* insertion/deletion mutation and in other strains where noted. *ng1427* expression in other strains was measured with “NG1427 qPCR” primer/probe set that detects full-length *ng1427* transcript where noted. Fold changes were determined using the comparative $2^{\Delta\Delta CT}$ while relative message expression was calculated using the $2^{\Delta CT}$ method (Schmittgen & Livak, 2008).

RecA-promoted in vitro NG1427 cleavage assay

This assay was adapted from the established LexA repressor cleavage assay (Gruenig *et al.*, 2008). Reactions were carried out at 37oC in solutions containing 25 mM Tris-OAc (80% cation, pH 7.4) 1 mM DTT, 5% (w/v) glycerol, 3 mM postassium glutamate, 3 mM $Mg(OAc)_2$, and an ATP regeneration system (2 mM phosphoenolpyruvate, 10 units/mL pyruvate kinase). The $RecA_{Ng}$ or $RecA_{Ec}$ proteins (3 μM) were preincubated with 9 μM φX174 circular ssDNA (NEB) for 5 minutes. ATP (3 mM) and the appropriate matched SSB protein (SSB_{Ec} or SSB_{Ng}) was added to 0.9 μM and the reactions were incubated a further 5 minutes. NG1427 protein or the NG1427-G113D protein (3 μM) was added to initiate the reaction, which continued for 45 minutes. Laemmli protein sample buffer (250 mM Tris-Cl pH 6.8, 4% SDS, 20% w/v glycerol, 10% β–mercaptoethanol, and 0.1% w/v bromophenol blue) was added to stop the reaction. Samples were subjected to SDS-PAGE electrophoresis on 17% acrylamide gels and stained with Coomassie Brilliant Blue to visualize protein.

EMSA

The intergenic regions of *ng1427/ng1428* and *recN* were amplified with primers specific to the intergenic regions via PCR by KOD polymerase (Novagen). The resulting PCR products

were purified using Qiaquick kit (Qiagen) and labeled with biotin using the Biotin 3' End DNA Labeling Kit (Pierce). Purified NG1427-G113D was added at 100 nM final concentration to a binding buffer containing a final concentration of 35 mM Tris, 20.25 mM NaCl, 150 mM KCl, 1 mM DTT, 50 ng/μl Poly (dI-dC), 0.05% NP-40, 2.7% glycerol, 5 mM $MgCl_2$; pH 7.5 and incubated for 20 minutes at 25°C. 40 fmol (final concentration 2 nM) of biotin-labeled DNA was then added and incubated for an additional 20 minutes at 25°C. Where indicated H_2O_2, NaOCl, $ONOO^-$, degraded $ONOO^-$ (Fisher)or MMS was added and incubated for 15 minutes at 25°C. The EMSA reaction was run out on a 5% PAGE gel (Bio-Rad) in chilled 0.5X TBE buffer and detected according to the LightShift Chemiluminescent EMSA Kit (Pierce) instructions.

Western Blot

Gc were grown to mid-log phase in liquid culture. Following a 1:10 dilution into amended GCBL (the $ONOO^-$ sample lacking Sodium Bicarbonate), the cultures were incubated for 15minutes (5 mM H_2O_2, 3 mM $ONOO^-$, 1.7 mM NaOCl), 30 minutes (25 ng/μl MMC) or 60 minutes (0.2% v/v MMS) at 37°C, or left untreated After 30 seconds, spectinomycin was added to a final concentration of 20 ng/ml to inhibit protein synthesis. An aliquot was then taken from each culture, spun down at ~15,000g for one minute, and stored on wet ice. The cell pellets were resuspended in 125 μl PBS, and quantified with BCA (Pierce). 3 μg of total cellular protein was loaded onto a 20% SDS-PAGE gel and subjected to electrophoresis. Western blot analysis was performed as previously described (Helm *et al.*, 2007), with 1:5000 anti-HA primary antibody (Roche) and 1:5000 horseradish peroxidase-conjugated anti-rat secondary antibody (Boehringer Mannheim). The signal was recorded using a chemilluminescent imager (Bio-Rad).

Detection of oxidized cysteine

NG1427-G113D was diluted 1/10 in protein storage buffer (25 mM Tris, 150 mM NaCl, 20% glycerol) with a pH of 7.0. NG1427-G113D was then added to a final concentration of 100 nM, and where applicable, 15 mM H_2O_2, 5 mM $ONOO^-$, or 20 μM NaOCl was added to the reaction. After a 15 minute incubation, 25-molar excess (2.5μM) of maleimide-PEG11-biotin in DMSO was added to the reaction and incubated for 16 hours at 25°C. The reactions were then split into two, with 1 μl of β–mercaptoethanol added to one 25 μl reaction and the other aliquot left untreated. 5X protein loading buffer lackingβ–mercaptoethanol was added, and samples were loaded onto 4–20% PAGE gels (Bio-Rad). Protein gels were stained with Coomassie Brilliant Blue. For detection of the biotin label, the proteins were transferred to a PVDF membrane (Millipore) and detected according to the LightShift® Chemiluminescent EMSA Kit instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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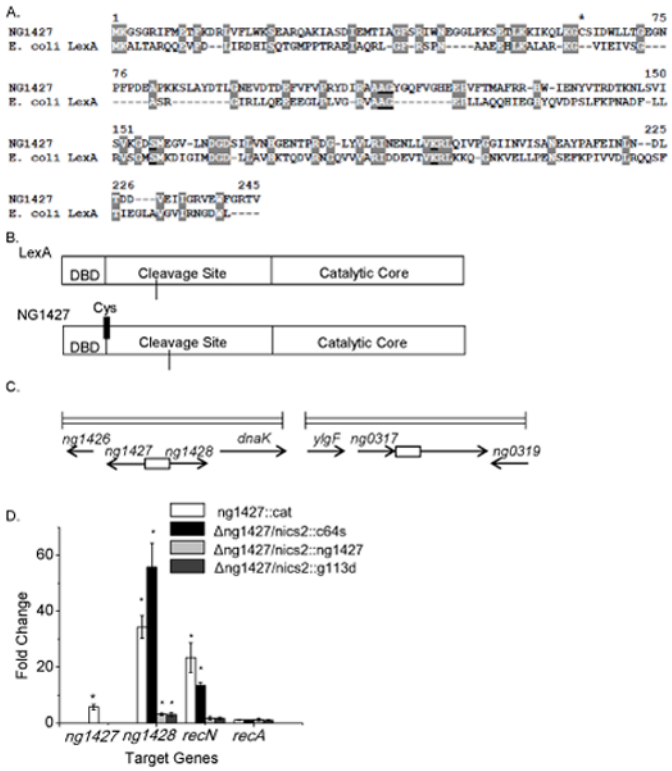

Figure 1. NG1427 is a transcriptional repressor that shares similarity to *E. coli* LexA

A: Amino acid sequence alignment comparing NG1427 and *E. coli* LexA. S24 peptidase cleavage and active sites are underlined and conserved residues are highlighted. Asterisk denotes single cysteine residue found in NG1427. B: Cartoon depiction of NG1427 and LexA protein domains. “DBD,” refers to the DNA binding domain. Location of single cysteine (Cys) in NG1427 is marked with black line. C: Diagram of the *ng1427/ng1428* and *recN* chromosomal loci in FA1090 Gc. Boxes denote intergenic regions containing putative promoters of *ng1427/ng1428* or *recN*. D: Fold change of target gene expression in *ng1427::cat*, Δ*ng1427/nics2::c64s*, Δ*ng1427/nics2::g113d*, and Δ*ng1427/nics2::ng1427*, relative to the parental strain as measured by qPCR with *omp3* used as a reference gene. Asterisks denote $p<0.05$ as calculated by Student’s *t*-test relative to negative control (*recA*). Error bars +/− s.e.m. All data shown are the result of at least 3 biological replicates (n=3).

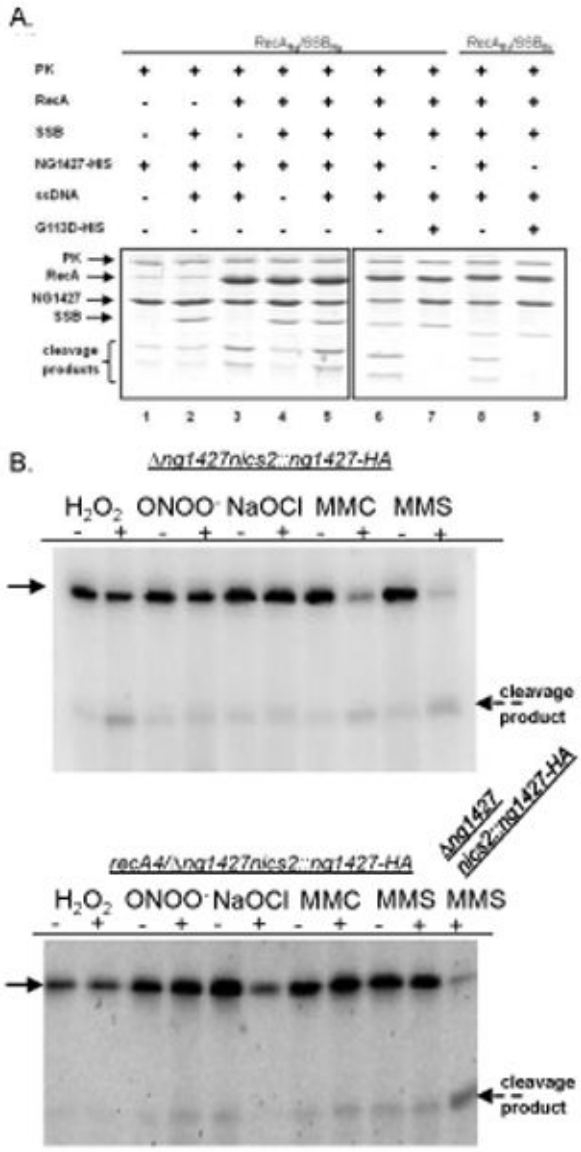

Figure 2. NG1427 undergoes RecA-mediated autoproteolysis *in vitro*, but limited *in vivo* autoproteolysis following oxidative treatment

A: Examination of RecA-facilitated autoproteolysis of NG1427 *in vitro*. Purified NG1427-HIS or NG1427-G113D-HIS protein was incubated with either $RecA_{Ng}$ or $RecA_{Ec}$ protein in the presence of the appropriate matched SSB protein, ssDNA, and an ATP regeneration system for 45 min. When a protein component (e.g. RecA or SSB) (lanes 2 and 3, respectively) was omitted as a control, the reaction was mock-treated with the storage buffer for the protein in question; when ssDNA was omitted, the reaction was mock-treated with TE storage buffer. Reactions were stopped and visualized on a 17% SDS-PAGE stained with Coomassie Brilliant Blue. The two NG1427 cleavage products are visible at the bottom of the gel. Gels are boxed to indicate that they were run on separate days. PK indicates pyruvate kinase.B: Examination of NG1427-HA turnover *in vivo* following treatment with oxidative and non-oxidative DNA damaging agents. Gc expressing NG1427-HA were treated with H_2O_2, $ONOO^-$, NaOCl, MMC, or MMS, and cell lysates were immunoblotted for the HA tag. Thick arrow points to full-length NG1427-HA protein, dotted arrow denotes

NG1427-HA C-terminal cleavage product with HA tag. Upper blot is NG1427-HA turnover in RecA^{+} cells, lower blot is NG1427-HA turnover in RecA^{-}cells.

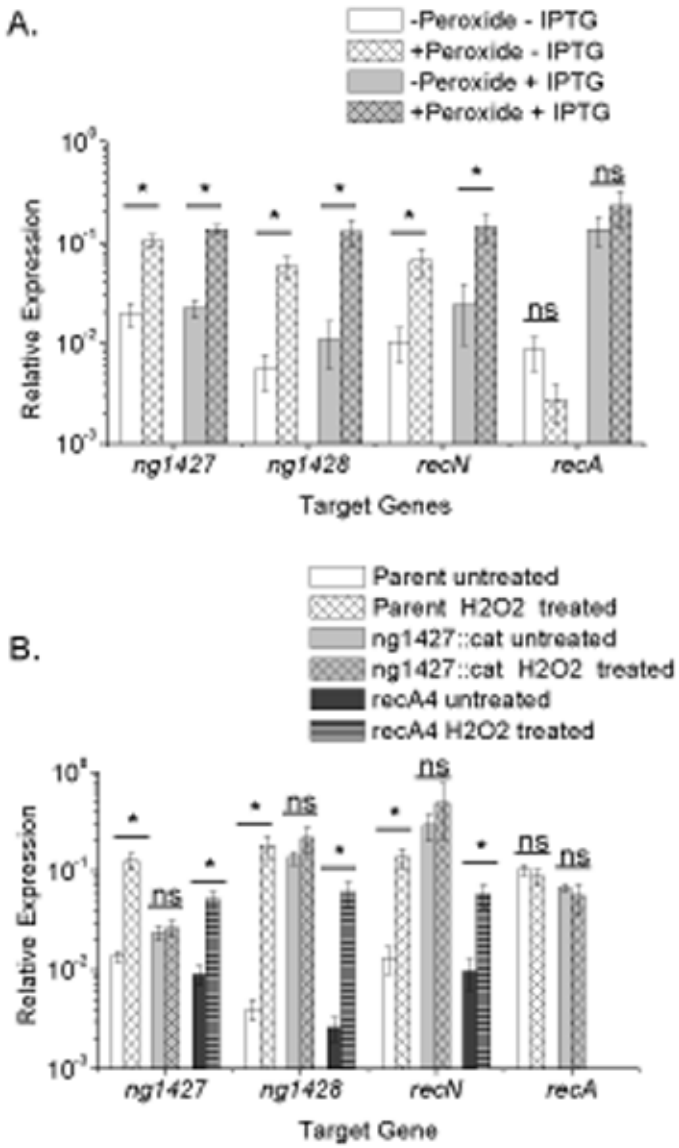

Figure 3. NG1427 is inducible following treatment with DNA damaging agents H_2O_2, and does not require RecA

All data show relative expression of each target gene calculated using a control transcript *omp3* for each condition tested. Error bars are +/− s.e.m, Asterisks denote $p<0.05$ as calculated by Student's *t*-test. A: H_2O_2 treatment induces the NG1427 regulon in a recA-independent manner. Induction of the NG1427 regulon following treatment with 5 mM H_2O_2 was measured in *recA6* Gc with or without IPTG induction. *ng1427* transcript was measured with the "*ng1427* probe" for each strain. B: The absence of RecA does not affect de-repression of the NG1427 regulon following H_2O_2 treatment. Induction of the NG1427 regulon following peroxide treatment was measured in parental, *ng1427::cat*, and *recA*4 Gc. *ng1427* transcript was measured with the "*ng1427* qPCR probe" for parental and *recA4* strains, and the "*ng1427::cat* 5' end qPCR probe" for the *ng1427::cat* strain. All data show a minimum of 3 biological replicates (n=3).

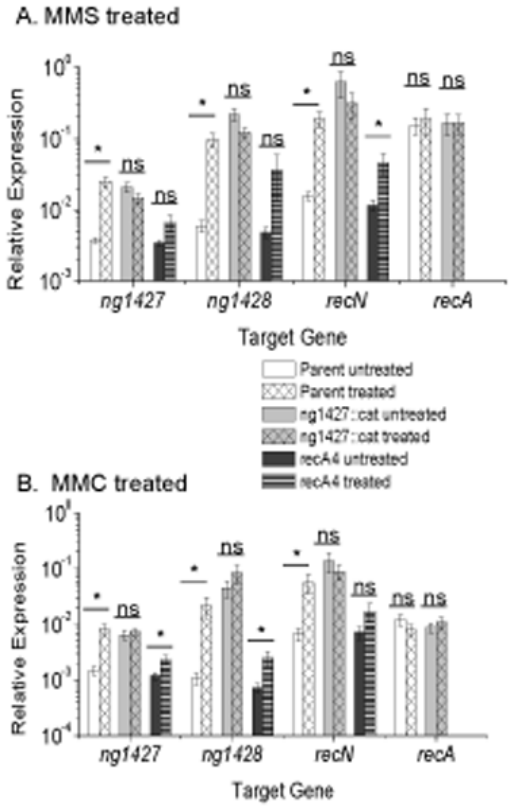

Figure 4. NG1427 requires RecA for full regulon induction following treatment with DNA damaging agents MMC and MMS

All data show relative expression of each target gene calculated using a control transcript *omp3* for each condition tested. Error bars are +/− s.e.m, Asterisks denote $p<0.05$ as calculated by Student's *t*-test. A: Full de-repression of the NG1427 regulon following MMS treatment requires expression of RecA. Induction of the NG1427 regulon after exposure to 0.02% (v/v) MMS was measured in parental, *ng1427::cat*, and *recA4* Gc. *ng1427* transcript was measured with the "*ng1427::cat* 5' end qPCR probe" for all strains. B: Full de-repression of the NG1427 regulon following MMC treatment requires expression of RecA. Induction of the NG1427 regulon after exposure to 25 ng/ml conc. MMC was measured in parental, *ng1427::cat* and *recA4* Gc. *ng1427* transcript was measured with the "*ng1427::c*at 5' end qPCR probe" for all strains. All data show a minimum of 3 biological replicates (n=3).

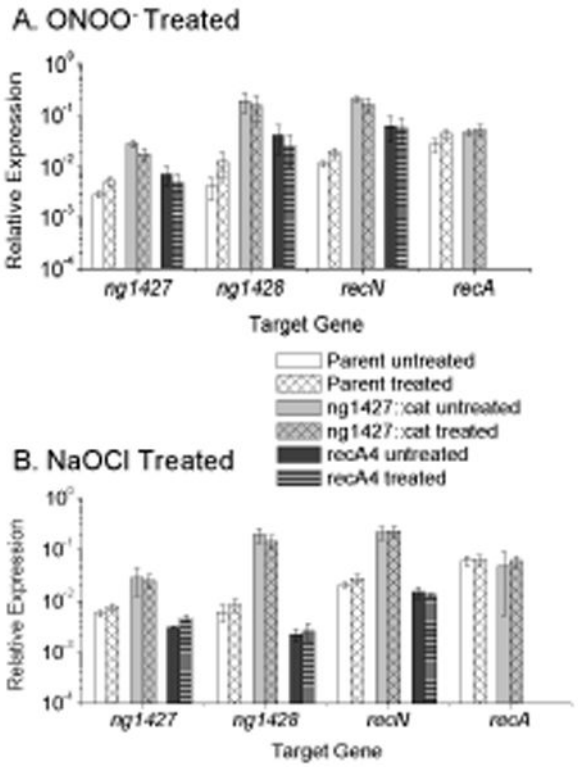

Figure 5. Treatment with ONOO− and NaOCl does not meaningfully affect the NG1427 regulon

All data show relative expression of each target gene calculated using a control transcript *omp3* for each condition tested. Error bars are +/− s.e.m, Asterisks denote $p<0.05$ as calculated by Student's *t*-test. A: $ONOO^-$ treatment does not strongly induce de-repression of the NG1427 regulon. Induction of the NG1427 regulon following exposure to 3 mM conc. $ONOO^-$ was measured in parental, *ng1427::cat* and *recA4* Gc. *ng1427* transcript was measured with the "*ng1427::cat* 5' end qPCR probe" for all strains. B: NaOCl treatment does not strongly induce de-repression of the NG1427 regulon. Induction of the NG1427 regulon following exposure to 1.7 mM conc. NaOCl was measured in parental, *ng1427::cat* and *recA4* Gc. *ng1427* transcript was measured with the "*ng1427::cat* 5' end qPCR probe" for all strains. All data show a minimum of 3 biological replicates (n=3).

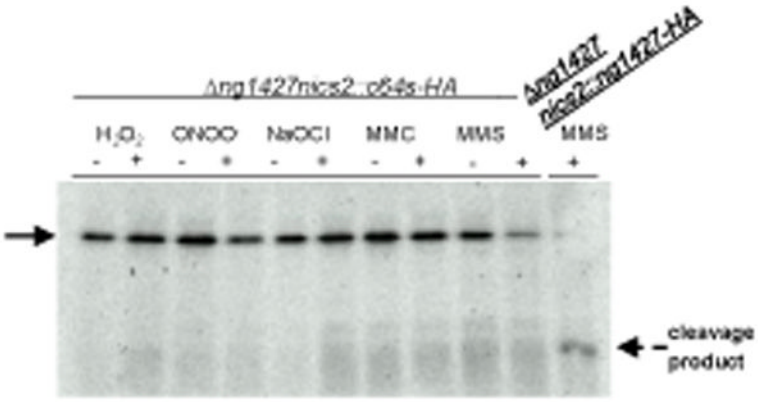

Figure 6. Disrupting cysteine 64 (Cys64) prevents RecA-mediated NG1427 autoproteolysis

Western analysis of strain NG1427-C64S-HA following treatment with either H_2O_2, $^{-}ONOO^{-}$, NaOCl, MMC, or MMS. Treatment of NG1427-HA was done in parallel and is included as a positive control Arrow points to full-length protein, dotted arrow denotes NG1427-HA C-terminal cleavage product with HA tag.

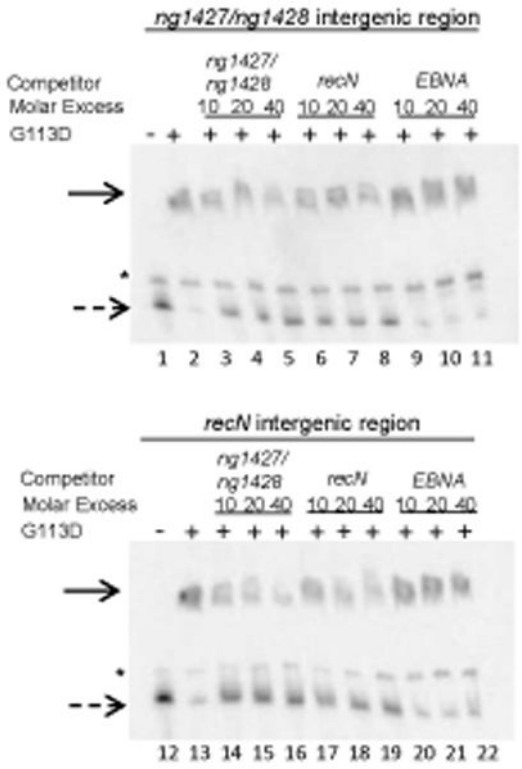

Figure 7. NG1427 binds specifically to the putative promoter regions of the NG1427 regulon

2 nM biotin-labeled DNA consisting of the *ng1427/ng1428* intergenic region or the upstream *recN* intergenic region was incubated with 100 nM of NG1427-G113D and unlabelled competitor DNA in molar excess denoted. “EBNA,” Epstein-Barr nuclear antigen target DNA, is an irrelevant piece of DNA used as a negative control. Reactions were then separated by electrophoresis on a 5% acrylamide Tris-HCl gel. The solid arrow denotes NG1427 bound DNA. The dotted arrow denotes unbound DNA. The asterisk denotes a non-specific band found in all reactions.

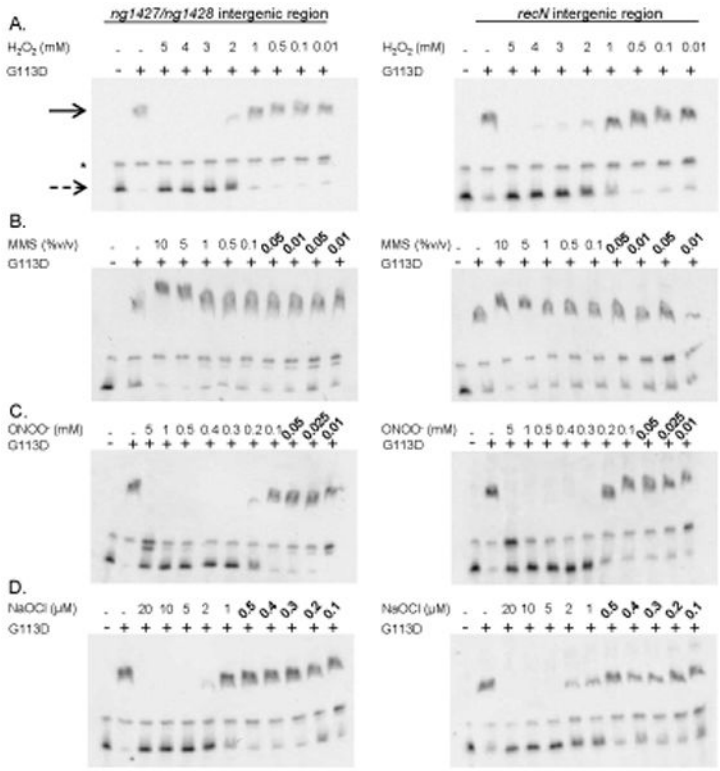

Figure 8. NG1427 cannot bind DNA following oxidative treatment

− 2 nM biotin-labeled DNA consisting of the *ng1427/ng1428* intergenic region or the upstream *recN* intergenic region was incubated with 100nM of NG1427-G113D. Where indicated, NG1427-G113D was either left untreated or treated with a varying range of concentrations of A: H_2O_2, B: MMS, C: $ONOO^-$, or D: NaOCl and incubated for 15 minutes following addition of DNA. The reaction was run on a 5% acrylamide Tris-HCl gel, transferred to a nylon membrane, and detected with streptavadin-HRP. The solid arrow denotes NG1427-G113D-bound DNA. The dotted arrow denotes unbound DNA. The asterisk denotes a non-specific band found in all reactions

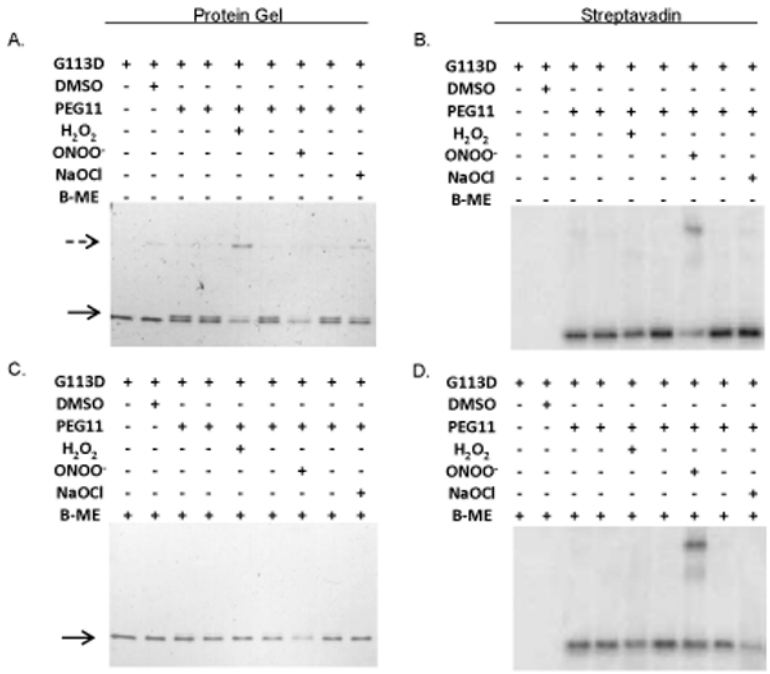

Figure 9. NG1427 is directly and reversibly oxidized

A: Direct visualization of the oxidation of NG1427. 100 nM of NG1427-G113D-HIS was incubated with H_2O_2, $ONOO^-$, or NaOCl for 15 minutes, then incubated with maleimide-PEG11-biotin. The reactions were then visualized on a non-reducing 4–20% SDS-PAGE gel stained with Coomassie Brilliant Blue. B: Determination of putative NG1427-G113D-HIS dimers free sulfhydryls groups. An equal volume aliquot from (A) was run out on a non-reducing 4–20% SDS-Page gel, transferred to a PVDF membrane, and detected with streptavadin-HRP. C: Examination of reversibility of disulfide-dimers of NG1427-G113D. 100 nM of NG1427-G113D-HIS was incubated with H_2O_2, $ONOO^-$, or NaOCl for 15 minutes then incubated with maleimide-PEG11-biotin. B-mercaptoethanol (B-ME) was then added to the reactions and were subsequently then visualized on a 4–20% SDS-PAGE gel stained with Coomassie Brilliant Blue. D: Detection of NG1427-G113D dimers following reduction. An equal volume aliquot of B-ME treated samples from (C) were run out on 4–20% SDS-Page gel, transferred to a PVDF membrane, and detected with streptavadin-HRP. The dotted arrow denotes the formation of the higher-molecular weight NG1427-G113D-HIS band, and the lower arrow denotes the mobility shift of NG1427-G113D-HIS following treatment with maleimide-PEG11-biotin.