OxyR tightly regulates catalase expression in Neisseria meningitidis through both repression and activation mechanisms

OxyR tightly regulates catalase expression in *Neisseria meningitidis* through both repression and activation mechanisms.

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Summary

Mechanisms for coping with oxidative stress (OS) are crucial for the survival of pathogenic *Neisseria spp.* in the human host. In this study we describe the mechanism by which OxyR finely regulates the catalase gene (*kat*) in *N. meningitidis*. Detailed transcriptional analyses show that catalase is transcribed from a single promoter which is induced by H_2O_2 in an OxyR-dependent manner and two key cysteine residues are essential for this. OxyR also represses the *kat* promoter: *kat* expression in the null mutant is at a constitutive intermediary level higher than un-induced, but lower than H_2O_2-induced levels in the wild type.

Our data are consistent with a model in which OxyR binds to the *kat* promoter and exerts (1) repression of transcription in the absence of OS signal, possibly by occluding an UP-element in the promoter, and (2) activation of the promoter in response to OS signal. This direct double-edged mechanism may ensure tight regulatory control and catalase is synthesized only when needed. In addition, our results provide an explanation for the altered OS resistance phenotypes seen in *Neisseria* mutant strains where, paradoxically, the *oxyR* mutants are more resistant than the wild type in oxidative killing assays.

Introduction

Human pathogens such as *Neisseria meningitidis* are exposed to oxidative stress by reactive oxygen species (ROS), which may damage virtually all biomolecules, generated as a natural bioproduct of aerobic respiration as well as during interactions with phagocytic cells, and H_2O_2-producing competing microflora. Basal defences are sufficient to cope with that amount of stress during routine aerobiosis. Instead, oxidative-response regulons, are activated whenever cells encounter environments containing exogenous H_2O_2 (Mongolsuk and Helmann, 2002; Storz and Zheng, 2000). Because H_2O_2 readily crosses membranes, cells that are exposed to these H_2O_2 sources must

defend themselves against the stress that ensues. Bacteria respond to oxidative stress by expressing genes required for the detoxification of reactive molecules, such as catalase and superoxide dismutase, which reduce H_2O_2 and superoxide respectively, as well as for the repair and maintenance of homeostasis.

Redox-sensing transcription factors are employed to sense elevated levels of ROS and activate the expression of antioxidant genes. The *Escherichia coli* OxyR transcription regulator, a member of the LysR family of bacterial transcription factors, is a prototype for proteins whose function is activated by ROS modification (Storz *et al*., 1990). During normal growth, OxyR is reduced and acts as a repressor of a subset of genes (Zheng *et al*., 1998). Upon exposure to elevated levels of H_2O_2, OxyR is oxidized and activates the expression of a regulon of genes encoding defence activities (Zheng & Storz, 2000). The initial reaction of OxyR with H_2O_2 is postulated to occur at Cys-199, leading to the formation of a Cys–sulphenic acid (Cys–SOH) intermediate which then reacts with Cys-208 to form an intramolecular disulphide bond (Zheng *et al*., 1998). Oxidized OxyR then activates transcription of antioxidant genes, including *katG* (encoding hydroperoxidaseI), *ahpCF* (encoding an alkyl hydroperoxide reductase), *dps* (encoding a non-specific DNA binding protein), *gorA* (encoding glutathione reductase), *grxA* (encoding glutaredoxin I), and *oxyS* (encoding a small regulatory RNA) (Storz and Zheng, 2000).

The OxyR protein contains a helix-turn-helix DNA-binding motif (HTH) in its N-terminal domain, and an oligomerization domain in the C-terminal domain, which also contains the two regulatory cysteine residues. OxyR forms a tetramer in solution and binds at extended binding sites with limited homology through four contact points (Toledano *et al*., 1994). Both the oxidized and reduced forms of OxyR bind DNA, however the different redox forms of the tetrameric transcription factor make different contacts along the promoter, enabling it to act as both an activator and a repressor (Toledano *et al*., 1994). Crystallization of both the oxidized and reduced forms of the protein have shown that disulfide bond formation between the two conserved cysteine residues in the oxidized form results in a significant structural change in the regulatory domain

(Choi *et al*., 2001), resulting in a repositioning of the protein on the DNA and triggers the transcriptional activation thought to occur via direct contact with RNA polymerase (Tao *et al*., 1993; Wang *et al*., 2006). It has been shown to function as a repressor of its own expression (*oxyR* gene transcription) and the Mu phage *mom* operon (Toledano *et al*., 1994). The C199S mutant form of the OxyR protein is locked in the reduced form, however, it is still active in DNA-binding activity and capable of repression of the *oxyR* and Mu phage *mom* operon gene promoters (Sun and Hattman, 1996; Kullik *et al*., 1995). Furthermore, by asymmetrically repositioning its contacts, OxyR can also impose opposite regulatory effects on a divergent promoter and thus function as a repressor of *oxyR* and an activator of *oxyS* from the same binding site (Toledano *et al*., 1994).

Studies of the pathogenic *Neisseria* suggest that their response to oxidative damage is fundamentally different from that of *E. coli.* In the pathogenic *Neisseria* spp., transcription of the catalase gene is induced in response to H_2O_2 (Stohl *et al*., 2005, Griffantini *et al*., 2004). However, it was found that in *N. gonorrhoeae* an *oxyR* mutant strain has considerably higher levels of catalase expression and is significantly more resistant to H_2O_2 killing than is the wild type (Tseng *et al*., 2003). Indeed, the authors show that the *oxyR* mutant has fourfold-higher catalase activity than the maximally induced wild type levels and as such they conclude that catalase expression is repressed by OxyR and is induced by H_2O_2 via OxyR derepression. This is distinct from the situation in *E. coli* and *Salmonella enterica* serovar Typhimurium, in which OxyR is a positive regulator of H_2O_2 inducible genes and in which increased sensitivity to hydrogen peroxide is seen in *oxyR* mutants (Christman *et al*., 1985, 1989). However, *N. gonorrhoeae* OxyR can complement an *E. coli oxyR* mutant and restore H_2O_2 resistance, implying that it can behave as an activator of ROS defence genes in *E. coli* (Tseng *et al*., 2003). In addition, a recent microarray study, revealed a rather limited regulon for OxyR in *N. gonorrhoeae*, with only two other deregulated genes in the *oxyR* mutant and these genes, *prx* and *gor*, were seen to be activated by the neisserial OxyR protein in response to the H_2O_2signal (Seib *et al*., 2007). Intriguingly, both of the newly identified OxyR target genes as well as *oxyR* itself were demonstrated to have a major role in biofilm production in *N. gonorrhoeae*.

The apparent contradictive role of the neisserial OxyR regulator in catalase expression remains unexplained. Therefore, we undertook a detailed analysis of transcriptional regulation of the catalase gene in *N. meningitidis* and the mechanism of action of the OxyR protein which is the subject of this study.

Results

Transcription of the catalase gene in response to H_2O_2

The catalase (*kat*) gene in *Neisseria meningitis* maps upstream and divergently oriented from the *rpoN* gene encoding for the alternative sigma factor σ^{54} (Fig. 1). In order to begin to study the transcriptional regulation of the *kat* gene, we first performed experiments to map the transcriptional start site and locate the promoter(s). Total RNA was prepared from a logarithmic phase culture of the wild type MC58 strain either untreated or treated with 150 μM H_2O_2 for 12 min, and primer extension with a *kat*-specific primer was carried out. The results of the primer extension analysis (Fig. 2A) show two major elongated primer products (B_1 and B_2) in the sample treated with H_2O_2 at positions corresponding to 143 and 32 nucleotides from the ATG start codon of the *kat* gene, respectively. A similar result was obtained by S1 nuclease protection assays with a radioactive *kat*-specific probe (data not shown), suggesting that these bands represent in vivo 5' ends of the *kat* mRNA. Analysis of the sequence upstream of the slow migrating band B_1 revealed –10 (TATAGT) and –35 (TTTTAA) elements with significant homology to the *E. coli* σ^{70} consensus promoter and this was defined as a likely start point of transcription and was called P_{kat}. The sequence surrounding the stronger fast migrating band B_2 had no similarity to promoter-like sequences for sigma factors defined to date, and therefore may be due to initiation at a *Neisseria*-specific promoter sequence or it may be a 5' end of the *kat* mRNA which occurs *in vivo* by degradation or processing of the transcript starting at P_{kat}.

We performed a time course experiment exposing the MC58 wild type cells to different quantities of H_2O_2 for increasing times and monitored the steady state levels of the transcripts from the major 5' ends P_{kat} and B_2, by primer extension. Fig. 2B shows the results of urea-acrylamide gel electrophoresis, which are also represented in graphical form. From these results we see that there is a very fast H_2O_2 concentration-dependent induction of the P_{kat} transcript which does not accumulate within the cell but has a very rapid turnover. Instead the B_2 transcript shows strong induction at all concentrations of H_2O_2 with maximum levels reached in response to 135 μM H_2O_2, at which concentration the transcript also shows maximum accumulation over the 30 min of sampling. In general at B_2 there is overall higher induction levels with slower turnover than that of P_{kat}. Therefore, it would appear that the induction and accumulation of these two major transcripts are regulated differentially in response to H_2O_2 in the cell. Since we are measuring the steady state levels of mRNA in the cell, this regulation may be a function of transcriptional initiation or RNA turnover, or both. Furthermore, the total *kat* transcript, B_1 and B_2 (Fig. 2B), seems to be higher in cells exposed to 135 μM compared to cells exposed to 405 μM, suggesting that 135 μM is the optimum concentration to allow maximum catalase induction under these experimental conditions.

In order to determine if the major transcripts P_{kat} and B_2 result from initiation at differentially regulated promoters, we generated a *kat* promoter transcriptional fusion to the promoterless *gfp* gene (*kat::gfp*), and also a mutant promoter fusion in which the –10 hexamer (TATAGT) of the P_{kat} putative promoter was deleted (*k-10::gfp*) as described in the Experimental procedures section. These fusions were introduced into the *N. meningitidis* MC58 genome at a site between the NMB1074 and NMB1075 converging genes by double recombination, generating the MC-*kat::gfp* and MC-*k-10::gfp* strains. RNA was prepared from these strains before and after H_2O_2 treatment and primer extension analysis using a *gfp*-specific primer (Fig. 2C) showed H_2O_2 induction of the P_{kat} promoter from the wild type *kat*::*gfp* fusion, and furthermore induction of other faster migrating bands, including B_2 although it does not appear to be a major product. No extension products were detected in RNA extracted from the MC-*k-10::gfp* mutant promoter fusion after H_2O_2 induction,

suggesting that transcription was abolished when deletions affected the -10 region of the P_{kat} promoter, and consequently, that all other bands, in particular B_2, are P_{kat} dependent and do not represent other *kat* promoters but likely shorter transcripts due to *in vivo* degradation or processing. We conclude that the catalase gene is transcribed from a single σ^{70}-dependent promoter P_{kat} initiating at 143 bp upstream of the coding sequence. The transcription from the P_{kat} promoter is induced in response to oxidative stress such as H_2O_2 and leads to the accumulation of a shorter transcript initiating 32 bp upstream of the *kat* gene, likely due to RNA processing. The differential steady state levels of these transcripts may result from differential stabilities of the RNA variants in response to oxidative stress.

OxyR regulation of kat *transcription in response to* H_2O_2

Tseng and colleagues (2003) have reported that catalase expression is repressed by OxyR of *N. gonorroheae* and that induction of *kat* by H_2O_2 is due to OxyR derepression. The meningococcal and gonococcal OxyR proteins are highly conserved with only 2 amino acid substitutions. Although they diverge significantly from the *E. coli* OxyR protein prototype, having approximately only 35% amino acid conservation, the conserved cysteine residues for oxidative activation of the protein are present. To establish the role of OxyR in regulation of catalase in *N. meningitis*, we generated a knockout of the *oxyR* gene (NMB0173) in the MC58 genome, as indicated in Fig. 3A. We prepared RNA from cultures of the wild type and the Δ173 OxyR null mutant that were grown to log phase and then either untreated or treated with increasing concentrations of H_2O_2 and the levels of the *kat* transcripts were measured by primer extension. Figure 3C shows that in the wild type MC58 strain, untreated cells show no detectable catalase transcription (lane 1) and a H_2O_2-concentration dependent induction of the P_{kat} promoter and accumulation at B_2 (lane 2-4). In the OxyR mutant, transcription of the *kat* gene is deregulated as it is no longer H_2O_2-inducible; the transcript levels of both P_{kat} and B_2 are identical under all conditions (lanes 5-8). Moreover when compared to the wild type, transcripts are at a level higher than the uninduced (lane 1) and lower than the H_2O_2-induced

levels (lanes 2-4). Therefore, in the absence of OxyR, *kat* is transcribed at a constitutive intermediary level, while in the wild type there is an OxyR-dependent down-regulation of the transcript in the absence of the H_2O_2 signal and an OxyR-dependent induction of the transcript in response to H_2O_2.

Previous reports have shown that substitution of the conserved cysteines (C199 and C208) may lead to mutant forms of the OxyR protein with altered activation or repression activities. In particular the C199S mutant of *E. coli* is 'locked' in the reduced form, cannot be activated by H_2O_2 to activate transcription at induced promoters, but is still active in its repressive capacity (Kullik *et al*., 1995). Instead, mutants of C208 also have an altered phenotype but appear to be lowly constitutively active (Kullik *et al*., 1995; Lee *et al*., 2004). To obtain a better understanding of the regulation of transcription by OxyR, we constructed a series of mutant strains expressing either the wild type *oxyR* gene, for complementation of the null mutant, or mutant forms of the gene with substitutions in the conserved cysteine residues. To this end we introduced the *oxyR* alleles under the control of the P_{tac} promoter at a heterologous region in the genome of the Δ173 null mutant, as described in the Experimental procedures section and as indicated in Fig. 3A. We generated a complemented mutant strain, Δ173-C, expressing the wild type gene; and two other strains that express either the C199S or the C208A mutant forms of the OxyR protein, Δ173_C199S and Δ173_C208A, respectively. Western Blot analysis with anti-OxyR serum (Fig. 3B), shows a similar level of expression of the wild type or mutant alleles in the mutant strains (lanes 3-5), indicating that the mutant proteins were stably expressed in the cell. However, *oxyR* is expressed slightly less in the recombinant strains from the P_{tac} promoter than in the MC58 wild type (lane 1). RNA analysis of the Δ173_C strain (Fig. 3C), shows no detectable *kat* transcription in the untreated cells (lane 9) and a concentration dependent H_2O_2 induction of *kat* transcripts (lane 10-12), indicating that the strain is complemented for *kat* regulation. The transcription of *kat* in the Δ173_C199S strain is at a constitutive intermediate level similar to the null mutant suggesting that the C199S protein is completely inactive (lane 13-16). Finally, in the Δ173_C208A strain, while there is no detectable

kat transcription in the untreated cells, the induction of *kat* is clearly impaired in response to H_2O_2, suggesting that the C208A protein complements for the down-regulation of *kat* in the absence of signal but is impaired in activation in response to H_2O_2. We conclude that these complementation experiments show the role of OxyR in the down-regulation of *kat* in the absence of H_2O_2 and the induction of *kat* in the presence of H_2O_2 and furthermore, the two conserved cysteines are essential for this regulation.

Oxidative stress phenotypes of OxyR mutants of N. meningitidis

The increased H_2O_2 resistance reported for the *N. gonorrhoeae oxyR* mutant strain (Tseng *et al*., 2003) is presumably largely due to the increased catalase expression seen in the *oxyR* mutant strain as a *katA* mutant of *N. gonorrhoeae* is highly sensitive to H_2O_2 (Seib *et al*., 2004). *N. meningitidis* is reported to have considerably lower catalase production than *N. gonorrhoeae* (Archibal and Duong, 1986). In order to understand if OxyR mutant of meningococcus shows similar increase in oxidative stress resistance phenotype to gonococcus we tested the MC58 wild type, the Δ173 null mutant and its complemented Δ173_C strain in H_2O_2 killing assays. Results in Fig. 4A show that the Δ173 mutant is indeed more resistant to H_2O_2 killing than the wild type or the complemented mutant strain Δ173_C, suggesting that OxyR, paradoxically, appears to have a negative effect on the oxidative stress response of meningococcus.

Furthermore, we assessed the oxidative stress phenotypes of the strains expressing the mutant OxyR proteins, Δ173_C199S and Δ173_C208A. The Δ173_C199S showed a similar oxidative stress resistance profile to the null mutant whereas the Δ173_C208A was more sensitive and exhibited an oxidative stress response phenotype similar to the wild type (Fig. 4B). These results indicate that there is a correlation between the ability of OxyR to down regulate the *kat* promoter and the oxidative stress sensitivity of the strain tested; in that the wild type, the Δ173-C and Δ173_C208A strains exhibit an OxyR-dependent down-regulation of the *kat* promoter in the absence of H_2O_2

(Fig. 3C; lanes 1, 9 and 17) and a more sensitive phenotype in H_2O_2 killing assays. This corroborates the hypothesis that OxyR-dependent down regulation of the catalase gene under normal growth conditions leads to oxidative stress sensitivity of the meningococcus in vitro.

E. coli *versus* N. meningitidis *catalase regulation*

We generated a transcriptional fusion of the *E. coli* inducible *katG* gene by cloning the *katG* promoter upstream of the promoterless *gfp* gene generating fusion Ec*katG::gfp*. This fusion along with the *N. meningitis* wild type (*kat::gfp*) and mutant (K-*10::gfp*) *kat* promoter fusions were assayed directly in *E. coli* for *gfp* expression by quantitative primer extension analysis. In the *E. coli* background, RNA analysis by primer extension showed no significant transcription of the Ec*katG::gfp* fusion in the absence of H_2O_2 signal, but significant H_2O_2 induction from the *katG* promoter, as expected, in response to H_2O_2 (Fig. 5A). Analysis of the neisserial *kat* promoter in the *E. coli* background indicated that the *kat::gfp* fusion gave constitutive transcription from P_{kat} with or without H_2O_2 stimulation, and no *gfp* expression from the *k-10::gfp* mutant fusion (Fig. 5B). Furthermore, when we introduced the *E. coli oxyR* gene under the control of the P_{tac} promoter into the Δ173 *oxyR* null mutant, in complementation experiments, regulation of the *kat* gene was constitutively high, similar to the null mutant, indicating that the *E. coli* OxyR protein in the meningococcal background could not complement for *kat* gene regulation (Fig. 5C) This indicates that the OxyR protein of *E. coli* was able, as expected, to induce transcription of Ec*katG::gfp* fusion in response to H_2O_2 stimulation but was unable to activate P_{kat} of *N. meningitidis* in response to H_2O_2, although the promoter was constitutively active in the absence of the stimulus.

We performed reciprocal experiments with the Ec*katG::gfp* fusion in the neisserial background by introducing single copy fusions into the wild type MC58 and Δ173 genomes generating MC-*katG::gfp* and Δ173*katG::gfp* strains, respectively (Table 1), and monitoring *katG* promoter levels in these backgrounds before or after H_2O_2 treatment. We were unable to detect transcription from the *katG* promoter in either untreated or H_2O_2 treated cells independent of OxyR presence by primer

extension (Fig. 5A). These results suggest that the meningococcal OxyR was unable to activate the promoter in response to H_2O_2 induction and that the neisserial transcription machinery did not recognize the *katG* promoter without appropriate activation. We conclude that OxyR of *E. coli* activates *katG* in response to H_2O_2, however, the neisserial OxyR protein is incapable of complementing for H_2O_2-inducible activation of *katG* and the *katG* promoter remains off. In comparison, the neisserial OxyR plays a role in down-regulating the *kat* promoter in the absence of H_2O_2 and a role in its induction in response to H_2O_2. The *E. coli* OxyR is equally incapable of complementing for this regulation and *kat* remains constitutively expressed.

OxyR binds directly to the kat *promoter*

In order to determine if OxyR directly binds the *kat* promoter *in vitro* we overexpressed and purified a recombinant meningococcal OxyR protein. The *oxyR* gene was cloned into an expression vector in *E. coli*, and the protein was expressed and purified by Ni^{2+}-affinity chromatography by virtue of an N-terminally located histidine tag. The protein was purified under native conditions as detailed in the Experimental procedures section, and samples from the expression and purification steps of the recombinant OxyR protein are shown in Fig. 6A.

A radioactively labelled probe containing the *kat* promoter was incubated with increasing concentrations of the OxyR protein and submitted to DNase I digestion. The results of the DNase I footprinting experiments are shown in Fig. 6B. On addition of 833 ng or more of protein, a region of protection and hyper-sensitivity corresponding to an area from –28 to –67 with respect to the +1 transcriptional start site becomes clearly evident. This indicates that OxyR binds directly to the *kat* promoter at a specific binding site whose location is overlapping and upstream of the –35 hexamer. The positioning of the binding site is reflective of the *katG* promoter in *E. coli* which is activated by *E. coli* OxyR in response to oxidation (Fig. 6D). Our data suggest that oxidated OxyR is needed for full activation/induction of the *kat* promoter and indeed the positioning of the binding site identified

is compatible with the hypothesis of OxyR, upon oxidation, functioning directly as a transcriptional activator of *kat* transcription by interacting with RNA polymerase.

However, our data also indicate that in the absence of OxyR the basal constitutive level of expression of the promoter is up-regulated, therefore binding of OxyR may down regulate the promoter by masking an alternative enhancer sequence. Due to its location overlapping and upstream of the -35 hexamer and its high A/T content (85%), the OxyR-binding sequence may overlap an UP element in P_{kat}, i.e. a third promoter element bound by the C-terminus of the α-subunit of RNA polymerase to stimulate transcription (for review see Gourse *et al*., 2000). In order to challenge this hypothesis we performed footprinting studies with the RNA polymerase from *E. coli* and the DNA probe consisting of the *kat* promoter. Results show that increasing concentration of RNA polymerase result in protection of the promoter in two regions spanning from –38 up to +43 overlapping the essential promoter elements and also at an upstream region spanning from –38 to –66 (Fig. 6C). This demonstrates that the A+T rich sequence upstream plays a role in RNA polymerase binding *in vitro*. We conclude that both OxyR and RNA polymerase can bind the region upstream of the *kat* –35 hexamer, which may act as an UP-element of the P_{kat} promoter.

Discussion

There is a wide range of oxidative stress defences in *N. meningitidis* that are increasingly being recognized as playing an important role in virulence (Wilks *et al*., 1998, Griffantini *et al*., 2004). *N. meningitidis* normally inhabits the nasopharynx where it will encounter airway epithelial cells capable of generating measurable levels of both reactive oxygen and nitrogen species (Rochelle *et al*., 1998), and activated polymorphonuclear leukocytes, will generate substantial amounts of superoxide and H_2O_2 as part of their oxygen-dependent bactericidal mechanism (Archibald and Duong, 1986) with local H_2O_2 concentrations as high as 100 μM inside phagocytes (Park *et al*.,

2005). The chemical oxidation of compounds in cell environments can generate low-micromolar H_2O_2, whereas *N. meningitidis* will also encounter concentrations as high as 1 mM near H_2O_2-generating competing microorganisms in the human nasopharynx (Pericone *et al*., 2000). Furthermore, in some instances *N. meningitidis* can traverse the mucosal barrier into the blood stream and cause invasive septicemia and meningitis disease passing through various niches with different levels of environmental oxygen availability and reactivity. Therefore, the ability to orchestrate such a tight control on the production of the catalase enzyme in *N. meningitidis* is essential to sustain resistance to the fluctuating levels of H_2O_2 encountered *in vivo*.

In this study we clarify a discrepancy currently thought to exist between the role of OxyR as an activator of the oxidative stress response and its reported role as repressor of the *kat* gene in *Neisseria* spp. We clearly show that OxyR of *N. meningitidis* activates the catalase gene in response to elevated levels of H_2O_2. It binds to a region overlapping the –35 hexamer of the single σ^{70}-dependent promoter P_{kat}, a position consistent with its role as an activator, resulting in a very fast and strong activation of transcription initiation, likely to be through direct contacts with the RNA polymerase. The *E. coli* OxyR protein has been shown to activate promoters through contact with the C-terminus of the alpha subunit of RNA polymerase (Wang *et al*., 2006; Tao *et al*., 1993). We have shown that the two conserved cysteines C199 and C208 are essential for the activation of *kat* expression in response to elevated levels of H_2O_2, indicating that OxyR is converted into a transcriptional activator of P_{kat} by the oxidation of the sulfhydryl groups of two reactive cysteine residues. The transcript initiating at P_{kat} is cleaved very rapidly at the B_2 site and accumulates as a shorter transcript with presumable higher stability. Nonetheless, the kinetics of the induction and turnover of the *kat* transcripts are essentially very rapid. It is likely that once the catalase protein is produced, the local elevated H_2O_2 concentrations are quickly dissipated and the signal for oxidation of the activator is therefore removed. Furthermore, one of the other members of the neisserial OxyR regulon which is induced by H_2O_2 is the Gor protein, which may play a role in the reversible reduction of the OxyR protein (Seib *et al.,* 2007; Carmel-Harel and Storz, 2000).

In contrast to many other inducible promoters (for instance *katG*), which often have weakly conserved promoter elements that are not recognized by RNA polymerase in the absence of the activator protein, P_{kat} is not completely dependent on oxidated OxyR protein for transcriptional initiation. In the null mutant or indeed in the *E. coli* background, the promoter is expressed at a constitutive intermediate level, which is likely due to an UP-element that enhances basal level transcription from the promoter in the absence of a functional OxyR regulator protein. Meningococcus may use this mechanism to ensure adequate catalase production, and hence protection against OS in the host, even under conditions in which OxyR protein levels may be low and therefore not present on the *kat* promoter (Fig. 7).

In meningococcus, OxyR also down regulates the constitutive promoter activity in the absence of elevated levels of H_2O_2. The OxyR-dependent repression is observed under normal microaerophilic growth conditions, where OxyR may exist in the reduced form (Zheng *et al*., 1998), suggesting that *kat* is repressed by reduced OxyR directly by occlusion of the promoter to RNA polymerase, possibly through masking of the UP-element and/or the –35 hexamer. Through complementation experiments with mutant proteins, the C208A protein elicited repression of the *kat* promoter but was hugely impaired in H_2O_2-inducible activation, while the C199S protein is completely inactive for repression and activation of *kat* (Fig. 3). In *E. coli*, C199S mutants are 'locked' in the reduced form and mutation of C208 seems to render the OxyR slightly constitutively active (Kulik *et al*., 1995; Lee *et al*., 2004). However, in our experiments the C199S and C208A mutants did not elicit a H_2O_2-independent repression and activation of the *kat* promoter, respectively, and therefore, may not have the corresponding phenotypes to the *E. coli* analogues. The C208A protein, impaired for activation of *kat* was fully functional for repression of *kat* in the absence of elevated H_2O_2 and this corroborates a model of regulation in which OxyR binds the promoter of *kat* and represses when not in its activated form (Fig. 7). RNA polymerase can initiate transcription of the P_{kat} promoter utilizing the UP-element enhancer sequence in the absence of a functional OxyR protein. However, OxyR is present on the P_{kat} promoter under all conditions in the cell; it represses the promoter when

reduced by possibly masking the UP-element; and when oxidized it directly activates RNA polymerase through binding of alpha subunit to its activator domain (Fig. 7). Once the redox state of OxyR is reversed, the reduced form once again represses transcription. In this way, OxyR controls a fast and effective fine-tuning of catalase expression in response to changing levels of H_2O_2. This intricate mechanism may allow *N. meningitidis*, a host-adapted pathogen, to respond to the changing host environment.

Although in the *E. coli* model system OxyR tends to either activate or repress at a single promoter, OxyR from other systems has been shown to exhibit similar dual regulation at one promoter. In *Sinorhizobium meliloti* OxyR represses and activates the *katA* and *katB* catalase genes (Luo *et al*., 2005), and in *Xanthomonas campestris* the *ahpC* promoter is regulated by a similar dual control mechanism (Loprasert *et al*., 2000).

The reciprocal analysis of the *E. coli* and *N. meningitidis* OxyR proteins and their regulation of the *kat* or *katG* reporter fusions clearly show distinct differences between the *E. coli* and *Neisseria* systems. The *oxyR* analogues, although essentially performing the same function, are not interchangeable and do not complement for regulation of the other's catalase gene. The implications of these experiments are that the two OxyR proteins recognize different binding sequences in the respective *kat* or *katG* promoters. A consensus binding motif has been determined for the oxidized form of *E. coli* OxyR (ATAGntnnnanCTATnnnnnnnATAGntnnnanCTAT) (Toledano *et al*., 1994) and while the *katG* binding site exhibits a sequence with significant conservation to the consensus, the A/T rich nucleotide sequence of the neisserial OxyR binding site in the *kat* promoter varies considerably (Fig. 6D). Both natural and synthetic binding sites were seen to diverge from the *E. coli* consensus (Toledano *et al*., 1994, Zheng *et al*., 2001) and so it is possible that there is an overlap of sequences that may be recognized by both proteins. The previous report of the ability of the gonococcal OxyR protein to complement the *E. coli oxyR* mutant for its H_2O_2 resistance phenotype may have been due to the induction of other oxidative stress defence genes and not the *katG* gene itself (Tseng *et al*., 2003). Furthermore, there is an essential difference in the two basal

catalase promoters. In the absence of its respective activator protein, the *E. coli katG* promoter is essentially off, whereas the neisserial promoter is constitutively active due to the dual action of the A/T rich OxyR binding site as probable UP element (Fig. 6D).

In the OxyR mutant, the constitutive activity of the catalase promoter in the absence of elevated levels of H_2O_2 is likely to be the main reason for the H_2O_2 resistant phenotype observed in the killing assay. Indeed, we correlate the ability to repress the *kat* promoter to a H_2O_2 sensitive phenotype in the site-directed OxyR mutant analyses (Fig. 4). We believe that while at low concentration of H_2O_2 wild type cells are able to induce the catalase promoter to a higher level than the *oxyR* mutant (Fig. 2B and Fig. 3), at high concentration of H_2O_2 (like those used in the killing assay) the cell homeostasis may be rapidly impaired, hindering also the synthesis of new catalase. In these experimental conditions, the OxyR deletion mutant and the C199S mutant have a clear advantage compared to the wild type strain and the C208A mutant, because of their constitutive catalase expression. Our results may explain the apparent paradox of increased resistance to OS in an OxyR mutant, as shown in this study and in the work by Tseng and co-workers (2003).

The physiological relevance as to why OxyR represses catalase is intriguing. It is tempting to suggest that this may have something to do with a graded response to the stress. Instead of an on/off redox regulation between oxidized (S-S) and reduced (S-H) forms of the OxyR protein, it has been shown that there may be a number of potential reaction intermediates to disulfide formation, including S-OH, S-NO, and S-SG which may have different activities (Kim *et al*., 2002). Under laboratory growth conditions without elevated levels of H_2O_2, meningococcal OxyR may not be present in the completely reduced form (OxyR-SH) but instead as a sulfenic acid, OxyR-SOH, with oxidation of a single cysteine, most likely C199. This form may have different activities on different promoters. It may bind and repress the *kat* promoter but activate other promoters such as that of the *prx* and *gor* genes, recently identified members of the regulon in gonococcus (Seib *et al*., 2007), which were indeed down-regulated in an OxyR mutant under laboratory growth conditions. Prx is a glutathione-based system that reduces H_2O_2, as well as alkylhydroperoxides, and is likely to

fulfil a role as major peroxidase for low concentrations of H_2O_2 in *Neisseria* as it does in *H. influenzae* (Pauwels *et al*., 2004), while the role of catalase is in the detoxification of elevated levels of H_2O_2 (Archibald and Duong, 1986). In this way, OxyR could process different redox-related signals into distinct functional responses and activate a tiered hierarchical response, first sending in the infantry and then the cavalry. This hypothesis of varying oxidation states of the protein having differential responsivity fits well with the phenotypes of the site-directed mutants on the *kat* promoter (Fig. 3) and furthermore, suggests that the OxyR regulon may be more extensive in *Neisseria* spp. than what was previously reported as the protein may not have been activated fully without the elevated levels of H_2O_2. Further characterization of the OxyR regulon of meningococcus will provide a better understanding of the coordinated response of *N. meningitidis* to oxidative stress and therefore its survival in the host.

Experimental procedures

Bacterial strains and culture conditions

The *N. meningitidis* strains used in this study are all derivatives of the MC58 sequenced strain (Tettelin *et al*., 2000) and are listed in Table 1. *N. meningitidis* strains were routinely cultured in GC-based (Difco) agar medium supplemented with Kellogg's supplement I (Kellogg *et al*., 1963) at 37ºC in a 5% CO_2-95% air atmosphere at 95% humidity. Strains were stocked in 10% skim milk and stored at –80°C. For liquid cultures, *N. meningitidis* strains were grown overnight on solid medium, resuspended in GC broth to an optical density at 600 nm (OD_{600}) of 1, and inoculated with a 1:20 dilution into GC broth supplemented with Kellogg's supplement I, 12.5 μM $Fe(NO_3)_3$, and, when required, erythromycin, kanamycin, and/or chloramphenicol added to achieve final concentrations of 5, 100, and 5 μg ml^{-1}, respectively. For transformation by naturally competent *N. meningitidis*, four to five single colonies of a freshly grown overnight culture were resuspended in

20 µl of phosphate buffered saline (PBS), spotted onto GC plates to which 5 to 10 µg of linearized plasmid DNA was added, allowed to dry, and incubated for 6 to 8 h at 37°C. Transformants were then selected on plates containing erythromycin (5 µg ml^{-1}), kanamycin (150 µg ml^{-1}) and/or chloramphenicol (5 µg ml^{-1}), and single colonies were restreaked on selective media for further analysis. Single colonies were resuspended in 50 µl of distilled water, placed in a boiling water bath for 5 min, and centrifuged in a bench top centrifuge for 5 min at 8,000 x *g*. One microliter of the sample was used as template for PCR analysis. *E. coli* DH5-α (Hanahan, 1983) and BL21(DE3) (Studier and Moffat, 1986) cultures were grown in Luria-Bertani medium, and when required, ampicillin, kanamycin and chloramphenicol were added to achieve final concentrations of 100, 25, and 30 µg ml^{-1}, respectively.

DNA techniques

DNA manipulations were carried out routinely as described by Sambrook *et al.* (1989). Small- and large-scale plasmid DNA preparations were carried out with a QIAprep Spin Mini kit and Plasmid Midi kit (QIAGEN, Inc.) according to the manufacturer's instructions. DNA fragments or PCR amplified products were purified from agarose gels with QiaEX DNA purification kit (QIAGEN, Inc,). PCR was performed in a Perkin-Elmer 2400 thermal cycler with Platinum *Taq* polymerase (Invitrogen). One microliter of each reaction mixture contained 10-50 ng of chromosomal DNA or 1 µl bacterial sample (see above), 100 pmoles of the required primers, and 200 µM concentration of each deoxynucleotide in a volume of 100 µl of 1x PCR buffer containing $MgCl_2$ (New England Biolabs, Inc.). After the initial denaturing step at 95°C for 5 min, 30 cycles of denaturing at 95°C, annealing at the appropriate temperatures for the specific primers, and elongation at 72°C were carried out. DNA fragments were routinely sequenced according to the dideoxy-chain termination method by using [α-^{32}P]dATP (NEN, Perkin Elmer) and a T7 sequencing kit (Pharmacia).

Expression and purification of recombinant OxyR

The *oxyR* gene was amplified from the MC58 genome using primers OxyR15f/OxyR15rev and cloned as a 860 bp EcoRV/BamHI fragment into the Klenow-blunted NdeI and BamHI sites of the pET15b+ (Invitrogen) plasmid, generating pET15b-OxyR. This plasmid was transformed into the *E. coli* BL21(DE3) strain and the expression of a recombinant OxyR protein containing a N-terminal histidine tag (His-tag), was induced by the addition of 1 mM IPTG, and the protein was purified by Ni-NTA (Qiagen) affinity chromatography under non-denaturing conditions according to the manufacturers' instructions. Protein concentrations of the OxyR protein was determined by the Bradford colorimetric method (Bio-Rad). We also expressed and purified other recombinant forms of the protein including a C-terminal His-tagged form and a GST-fusion form, however, neither of these proteins showed any DNA binding activity towards the *kat* promoter in *in vitro* binding experiments. Furthermore, the His-OxyR recombinant protein used in the current study exhibited a transient unstable DNA-binding activity, which was lost when stored at 4° or at -20ºC overnight. Therefore, the OxyR protein was purified from small scale induction cultures and was used immediately in binding reactions. To prepare anti-OxyR antiserum, 20 µg of purified protein was used to immunize 6-week-old CD1 female mice (Charles River Laboratories), and four mice were used. The protein was given intraperitoneally, together with complete Freund's adjuvant for the first dose and incomplete Freund's adjuvant for the second (day 21) and third (day 35) booster doses. Bleed-out samples were taken on day 49 and used in Western blot analysis.

Construction of catalase promoter transcriptional fusions

To generate transcriptional fusions of the promoters studied, a series of plasmids carrying catalase promoters fused to the *gfp* gene were constructed which could be assayed in *E. coli* and also used for allelic exchange in *N. meningitidis* strains at a chromosomal location between two converging ORFs, NMB1074 and NMB1075. A plasmid consisting of a promoterless *gfp* gene and the *ermAM* erythromycin resistance genes flanked by upstream and downstream regions for allelic replacement

was generated by substituting a 3.4kb SphI-BamHI fragment containing the *lacZ* gene in pSL-Fla-Ery (Ieva *et al*., 2005) with a 748 bp SphI-BamHI fragment generated by PCR amplification of the *gfp*mut1 (Cormack *et al*., 1996) allele using primers GFP-F and GFP-R (Table2). The wild type *kat* promoter from MC58 or the wild type *katG* promoter from *E. coli* (Triggs-Raine *et al*., 1988) were then amplified from the genomes of MC58 or DH5-α, respectively using the primer pairs KatPfw1/KatPrev3 and KatGFwd/KatGRev respectively, and cloned into pGEMT (Promega) generating pKat and pKatG plasmids respectively, and then subcloned as 338 bp or 250 bp NsiI-SphI fragments upstream of the *gfp* gene, generating p*kat::gfp* and p*katG::gfp*, respectively. The –10 hexamer (TATAGT) of the MC58 *kat* promoter was deleted in the p*kat::gfp* plasmid generating the p*k-10::gfp* transcriptional fusion plasmid. The deletion was performed using the Quik-Change kit (Stratagene, Inc) with the complementary primer pair K1-10F/ K1-10R. Transcription from these fusions was monitored in *E. coli* through green fluorescent protein (GFP) production, quantified by Western blot, and also by quantitative primer extension using a *gfp*-specific primer GPE2. Single copy fusions were also introduced into the MC58 genome by transformation of the p*kat::gfp*, p*katG::gfp* and p*k-10::gfp* plasmids and selection on erythromycin, and the resulting strains MC-*kat::gfp,* MC-*katG::gfp,* and MC-*k-10::gfp* (Table 1) were verified by PCR. Although no GFP was detectably produced in the neisserial background under the conditions described, the transcription from the promoters was analyzed by quantitative primer extension experiments with the GPE2 primer.

Construction of the OxyR mutant and complementation with the wild type or mutated oxyR *gene*

To construct an *oxyR* null mutant, the *oxyR* gene was replaced with a kanamycin cassette by allelic replacement through transformation of the MC58 strain with plasmid pG3- Δ173 which was generated as follows. Upstream and downstream flanking regions of *oxyR* were amplified from the MC58 chromosome with primer pairs 173FL1/173FL2 and 173FL3/173FL4 (Table 2) and cloned as 500 bp PstI/BamHI and 575 bp BamHI/EcoRI fragments, respectively, flanking a kanamycin

resistance 1.4-kb BamHI cassette from plasmid pILL600 (Labigne-Roussel *et al*., 1988) into the EcoRI-PstI sites of the pGem3Z (Promega) cloning vector backbone. Transformants were selected on kanamycin, and resistant colonies were analyzed by PCR for correct insertion by a double homologous recombination event resulting in an allelic replacement of the *oxyR* gene. This mutant was named Δ173 (Table 1).

In order to complement the *oxyR* null mutant, the *oxyR* wild type gene or *oxyR* mutated genes, were reinserted under the control of the P_{tac} promoter into the chromosome of Δ173 between the converging ORFs NMB1428 and NMB1429, through the transformation of the Δ173 strain with the pCOM173, pCOM-Oeco or pCOM-C199S or pCOM-C208A plasmids generated as follows. The P_{tac} promoter was amplified from plasmid pMMB206 (Morales *et al*., 1991) using the Tac1/Tac2 primer pair and cloned as a 140 bp KpnI-BamHI fragment into pSLComCmr plasmid (Ieva *et al*., 2005) consisting of a chloramphenicol resistance gene flanked by upstream and downstream regions for integration between the NMB1428 and NMB1429 ORFs, generating pComPtac. The wild type *N. meningitidis* or *E. coli oxyR* gene were amplified from the MC58 or DH5-α genome with primer pairs 173FWSD-Bam/173RV-Nsi and Oecom-Fw/Oecom-RV, respectively, and cloned as 875 bp or 988 bp BamHI/NsiI fragments downstream of the P_{tac} promoter generating pCOM173 and pCOM-Oeco, respectively. The pCOM-C199S and pCOM-C208A plasmids are derivatives of pCOM173, containing site-directed mutant alleles of the *oxyR* gene, in which the TGT cysteine-199 codon was substituted by the TCA serine codon, or the the TGT cysteine-208 codon was substituted by the GCA alanine codon, respectively. The mutations were introduced in the pCOM173 plasmid using the QuikChange kit (Stratagene, Inc) and the FW199S/REV199S and FW208A/REV208A (Table 2) primer pairs, respectively. The Δ173 strain was transformed with the pCOM173, pCOM-Oeco, pCOM-C199S and pCOM-C208A plasmids; transformants were selected on chloramphenicol; and resistant colonies were analyzed by PCR for correct insertion by a double homologous recombination event. The resulting strains were named Δ173_C,

Δ173_EcoOxy, Δ173_C199S, and Δ173_C208A respectively (Table 1). Expression of the meningococcal wild type and mutant OxyR proteins in the complemented strains was confirmed by Western Blot analysis with anti-OxyR serum raised in mice against the recombinant OxyR purified protein.

H_2O_2 treatment of N. meningitidis *culture and RNA preparation*

In order to analyze the transcriptional response of *N. meningitidis* culture to different concentration of H_2O_2, *N. meningitidis* strains were cultured to OD_{590} of 0.5 in 100 ml of GC medium, then aliquots of 20 ml were added to 50 ml Falcon tubes containing respectively 20 µl of water or H_2O_2 at the concentration of 0.015, 0.045, 0.135, 0.405 M. The tubes had a final H_2O_2 concentration of 0, 0.015, 0.045, 0.135, 0.405 mM, respectively, and were incubated at 37°C with shaking. After 12 min (if not indicated) or the number of minutes indicated, the culture aliquots were added to 15 ml of frozen media in order to immediately reduce the media temperature to 0°C. Subsequently, the cultures were centrifuged and total RNA was extracted as previously described (Spohn *et al*., 1997).

Primer extension analysis and S1 nuclease protection analysis

Total RNA extracted from *E. coli* or *N. meningitidis* cultures was used in primer extension experiments as previously described (Ieva *et al*., 2005). To ensure correct mapping of the promoter, sequencing reactions were carried out on the cloned promoter region with the same primer according to the dideoxychain termination method using [α-32P]-dATP (Amersham Biosciences) and a T7 sequencing kit (USB Corporation) and electrophoresed in parallel to the primer extension reactions. A radioactively labelled *kat*-specific probe was prepared and S1 nuclease protection assays were carried out as described previously (Grifantini *et al*., 2004).

DNase I footprinting

The *kat* promoter region was amplified from the MC58 genome with primers KatPrev2 and KatPfwd and cloned as a 208 bp BamHI-EcoRI fragment into pGemT (Promega) generating the pKat-s plasmid. A radioactive probe for DNA footprinting of the *kat* promoter was prepared as follows: Approximately 2 pmol of the pKat-s plasmid was linearized with BamHI, subjected to dephosphorylation using CIP (New England Biolabs, Inc.), and then 5' end-labelled using 5 pmol of [γ-^{32}P]ATP with T4 polynucleotide kinase (New England Biolabs, Inc.). The plasmid was then digested with EcoRI, and the *kat* promoter fragment, labelled at one extremity, was purified by preparative polyacrylamide gel electrophoresis as above. Protein-DNA complexes were formed in 50 µl of footprinting buffer (50 mM Tris-HCl [pH 8], 50 mM KCl, 10 mM $MgCl_2$, 0.01% NP-40, 10% glycerol) containing approximately 5 ng (20,000 cpm) of the labelled probe and a 200 ng of sonicated salmon sperm DNA as the non-specific competitor DNA and freshly prepared recombinant OxyR protein, or RNA polymerase of *E. coli* (Roche) in final concentrations as indicated, for 15 min at room temperature. Following incubation for 1 min at room temperature with 0.01 U of DNase I (Roche) and 5 mM $CaCl_2$, the reaction was stopped by addition of 140 µl of stop buffer (192 mM Na acetate, 32 mM Na_2EDTA, 0.14% SDS, 64 µg/ml of sonicated salmon sperm DNA). Samples were phenol extracted, ethanol precipitated, resuspended in denaturing sample buffer, and fractionated on urea–6% acrylamide gels.

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Figure Legends

Fig. 1. Schematic representation of the *kat* locus showing the upstream intergenic sequence and the positions of regulatory elements identified in this study. The P_{kat} promoter elements are underlined and in bold-face. *In vivo* 5' ends of RNA (B_1 and B_2) are indicated in bold-face. The OxyR binding site is shaded in grey. The transcriptional start site of the *rpoN* gene was determined by primer extension (data not shown) and is also indicated in bold with its putative promoter elements underlined. Levels of transcription at this promoter were unaffected by H_2O_2. Translational start sites for both *rpoN* and *kat* genes are in bold.

Fig. 2. Mapping of the transcriptional start site of the catalase gene. Total RNA was prepared from the MC58 wild type cells before (-)and after (+) exposure to 150 μM H_2O_2 and **(A)** hybridized to a *kat*-specific primer KatPE1 and reverse transcribed in primer extension analysis. The elongated primer products are indicated and the two major bands indicated as $B_1(P_{kat})$ and B_2, corresponding to the +1 transcriptional start site of the promoter and a highly abundant downstream 5' end, are highlighted. Minor bands which may correspond to less abundant 5' ends are indicated with asterisks. The GATC lanes show a sequencing reaction with the KatPE1 primer and the promoter region cloned into pKat (see Material and Methods) which was run in parallel for correct mapping of the bands. **(B)** A time course analysis of the response of the *kat* transcripts to increasing concentrations of H_2O_2 by primer extension analysis. Total RNA was prepared from the MC58 wild type culture grown to log phase and treated with increasing concentrations of H_2O_2 as indicated for the number of minutes indicated. Bands corresponding to the transcriptional start site of the promoter P_{kat} and the major downstream 5'end B_2 are shown. The steady state levels of each transcript were quantified using phosphoimaging and the Image Quant software and are shown underneath in graphical representation. **(C)** Fusion analysis of the *kat* promoter. The wild type *kat* promoter and a mutant promoter deleted in the putative –10 hexamer upstream of P_{kat} were cloned upstream of a promoterless *gfp* gene and introduced as a single copy fusion at a heterologous

location in the MC58 genome generating MC-*kat::gfp* and MC-*k-10::gfp* strains, respectively. Total RNA, prepared from logarithmic phase cultures before (-)and after (+)exposure to 150 μM H_2O_2 , was hybridized to a *gfp*-specific primer, GPE2 (Table 1), and reverse transcribed. The GATC lanes show a sequencing reaction with the GPE2 primer and the p*kat::gfp* plasmid (see Material and Methods) which was run in parallel for correct mapping of the bands.

Fig. 3. (A) Schematic diagram of the *oxyR* locus in MC58 and the construction of the isogenic mutant and complementing derivatives. **(B)** Immunoblot of OxyR protein levels in wild type and mutant derivative strains. Total protein corresponding to 10 μg of whole cell extract was separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with α-OxyR antiserum (top panel), or α-Fur antiserum (bottom panel) as a negative control. **(C)** RNA analysis of the *kat* promoter transcripts in the wild type and mutant derivative strains by primer extension analysis. Total RNA was prepared from indicated cultures grown to log phase and treated for 12 minutes with increasing concentrations of H_2O_2 as indicated, hybridized to the KatPE1 primer and reverse transcribed. The bands corresponding to the transcriptional start site of the promoter P_{kat} and the B_2 downstream 5'end are shown.

Fig. 4. (A) H_2O_2 killing assay of *N. meningitidis* strain MC58 wild type (WT), the Δ173 OxyR mutant strain and the Δ173_C complemented mutant strain. Log phase cells were resuspended in BHI broth and exposed to a final concentration of 4 mM H_2O_2. Samples were taken at 0, 20 and 60 minutes, cells plated and viable CFU determined. Experiments were performed in triplicate. Y-error bars indicate +/- 1 standard deviation of the mean **(B)** H_2O_2 killing assay of *N. meningitidis* strain MC58 wild type (WT), the Δ173 OxyR mutant, and the mutant derivative strains Δ173_C199S (C199S) and Δ173_C208A (C208A) expressing mutant OxyR proteins. Log phase cells were resuspended in BHI broth and exposed to a final concentration of either 0, 5 or 7.5 mM H_2O_2.

Samples taken at 15 minutes, cells plated and viable CFU determined. Experiments were performed in triplicate. Y-error bars indicate +/- 1 standard deviation of the mean.

Fig. 5. Fusion analysis of the *kat* and *katG* promoter. **(A)** Reporter analysis of the *E. coli katG* promoter (Ec-*katG::gfp)* fusion in the *E. coli*, MC58 and Δ173 null mutant backgrounds. Total RNA was isolated from log phase DH5-α cells carrying the p*katG::gfp* plasmid (*E. coli*); or from MC-*katG::gfp* (MC58): or Δ173-*katG::gfp* (Δ173) cultures before (-) or after treatment with the 405 μM (+) or 1 mM (++) of H_2O_2 and analyzed by primer extension using a *gfp*-specific primer GPE2. **(B)** Reporter analysis of the *N. meningitidis* catalase promoter fusions (Nm*kat::gfp*) in the *E. coli*. Total RNA was isolated from log phase DH5-α cells carrying the p*kat::gfp* plasmid (*kat*); or the p*k-10::gfp* plasmid (*k-10*); or from MC-*katG::gfp* (*kat*), or MC-*k-10::gfp* (k-10) cultures before (-) or after treatment with the 405 μM (+) or 1 mM (++) of H_2O_2 and analyzed by primer extension using a *gfp*-specific primer GPE2. **(C)** *E. coli* OxyR does not complement Δ173 for catalase regulation. Total RNA was isolated from the Δ173_EcoOxy cultures before (-) or after treatment (+) with the 405 μM of H_2O_2 and analyzed by primer extension using a *kat*-specific primer KatPE1.

Fig. 6. Purification and *in vitro* binding assays with recombinant OxyR. **(A)** SDS-PAGE showing steps from the expression and purification of a recombinant OxyR including: uninduced (-) and IPTG-induced (+) whole cell extracts of BL21 culture carrying the pET15b-OxyR plasmid construct expressing a His-tagged OxyR protein; the cleared soluble fraction (SF) and flowthrough (FT) after binding to the Ni_NTA column; and the eluted fraction (E) containing the His-tagged OxyR protein. **(B)** *In vitro* binding of the purified OxyR protein to the catalase promoter. A radioactive probe containing the *kat* promoter was prepared from plasmid pKat-s as a 208 bp EcoRI-BamHI fragment labelled at the BamHI site. The DNA probe was incubated with increasing amounts of OxyR protein: lanes 1-6; 0, 93, 278, 833, 2500, 7500 ng respectively; and subjected to

DNase I digestion. **(C)**. Binding of RNA polymerase to the *kat* promoter. The *kat* end-labelled probe was incubated with increasing amounts of RNA polymerase, lanes 7-12: 0, 2, 4, 6, 8, and 10 U of RNA polymerase. The vertical bars on the right side of each panel indicate the areas of DNase I protection, small arrows refer to DNase I hypersensitive sites. The numbers displayed indicate the position of the relevant nucleotide with respect to the +1 transcriptional start site. The G+A lane shows a sequence reaction on the DNA probe used as a size marker (Maxam and Gilbert, 1977). **(D)** Comparison of the nucleotide sequence and regulatory elements of the *kat* promoter of MC58 and the *katG* promoter of *E. coli*. The UP-elements of the rRNA gene promoters *rrnD* P1 and *rrnB* P1 are reported and aligned as in Estrem *et al*., (1998).

Fig. 7. Model of *kat* gene transcriptional regulation by OxyR in *N. meningitidis*.

TABLE 1. *N. meningitidis* strains used in this study

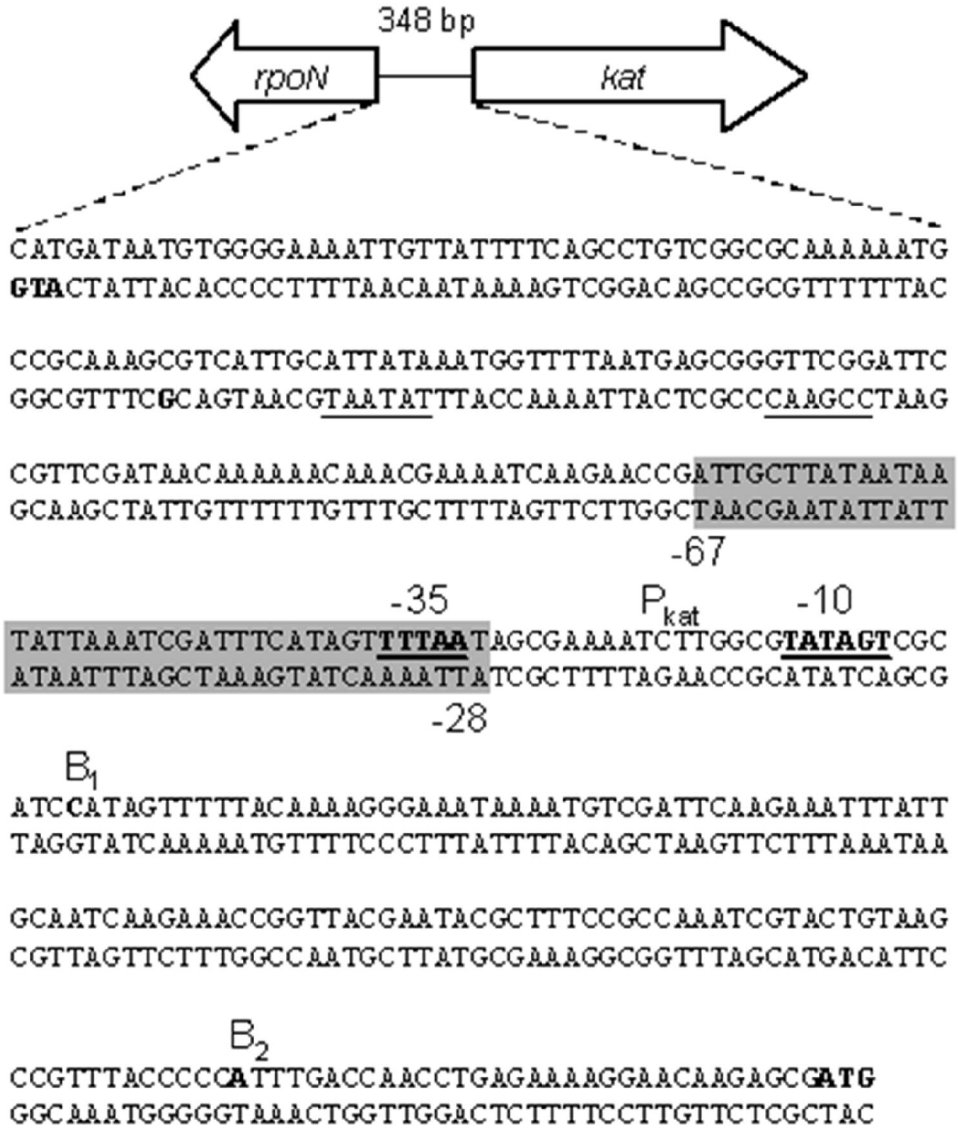
Strain	Description	Reference
MC58	Clinical isolate; sequenced strain	Tettelin *et al.* 2000
Δ173	OxyR null mutant derivative of MC58 in which the gene NMB0173 has been substituted by a kanamycin cassette, Km^R	This study
Δ173_C	Complemented OxyR mutant, derivative of Δ173 containing the NMB0173 gene under the control of the P_{tac} promoter between ORFs NMB1428 and NMB1429, Km^R , Cm^R	This study
Δ173_C199S	Derivative of Δ173 containing the NMB0173 gene, with the mutation C199S, under the control of the P_{tac} promoter between ORFs NMB1428 and NMB1429, Km^R , Cm^R	This study
Δ173_C208A	Derivative of Δ173 containing the NMB0173 gene, with the mutation C208A, under the control of the P_{tac} promoter between ORFs NMB1428 and NMB1429, Km^R , Cm^R	This study
Δ173_EcoOxy	Derivative of Δ173 containing the *E. coli oxyR* gene, under the control of the P_{tac} promoter between ORFs NMB1428 and NMB1429, Km^R , Cm^R	This study
MC-*kat::gfp*	Derivative of MC58 containing the promoter region of the *kat* gene fused to a promoterless *gfp* gene chromosomally located between the ORFs NMB1074 and NMB1075, Ery^r	This study
MC-*k-10::gfp*	Derivative of MC58 containing the mutant *kat* promoter lacking the –10 hexamer fused to a promoterless *gfp* gene chromosomally located between the ORFs NMB1074 and NMB1075, Ery^r	This study
MC-*katG::gfp*	Derivative of MC58 containing the promoter region of the H_2O_2-inducible *E. coli* catalase gene *katG* fused to a promoterless *gfp* gene chromosomally located between the ORFs NMB1074 and NMB1075, Ery^r	This study

TABLE 2. Oligonucleotides used in this study

Name oligo	Sequence[a]	Site[b]
173FL1	atatatctgcagCTGAATCCCAAAACGTCTTGC	PstI
173FL2	atatatggatccGACGTTCTTGGGCGACTGCG	BamHI
173FL3	atatatggatccCAGGCATTGTTTTCAAAACGCC	BamHI
173FL4	atatatgaattcCGTCGCCGTCTGCAACGGCG	EcoRI
173FWSD-Bam	atatatggatccAAGGTATAGGACATGACCTTAAC	BamHI
173RV-Nsi	atatatatgcatCTAGTCGCAGATAAAACTTACCC	NsiI
FW199S	CTGACGGAAGGCAACtcaATGCGGGATCAGG	
FW208A	CAGGTACTCTCAAGCgcaTCCGAATTGGCGGCGA	
GFP-F	attcagcatgctttaagaaggagatctactatgagtaaaggagaa	SphI
GFP-R	attcagggatccctatttgtatagttcatccatgcc	BamHI
GPE2	ggacaactccagtgaaaagttc	
K1-10F	GCGAAAATCTTGGCGCGCATCCATAGTTTTTAC	
K1-10R	GTAAAAACTATGGATGCGCGCCAAGATTTTCGC	
KatGRev	ttatggatatcgtctgacgtgc	
KatPrev2	atatatggatccGGCGGAAAGCGTATTCGTAACC	BamHI
KatPfwd	atatatgaattcTAATGAGCGGGTTCGGATTCC	EcoRI
KatPfw1	atatatatgcatGGGGAAAATTGTTATTTTCAG	NsiI
KatPrev3	attcagcatgcCGCTCTTGTTCCTTTTCTCAG	SphI
KatPE1	GGGTTACAGGGCATTTGGAGG	
KatGFwd	ttctccctcattacttgaagg	
Oecom-FW	attcaggatccgccatgaactatcgtggcgatg	BamHI
Oecom-RV	attcaatgcatcgatggcggaagcctatcgg	NsiI
OxyR15f	atatatgatatcACATGACCTTAACCGAATTGCG	EcoRV
OxyR15rev	atatatggatccGATCAGCGTCTATTTTGAATGG	BamHI
REV199S	CCTGATCCCGCATTgaGTTGCCTTCCGTCAG	
REV208A	TCGCCGCCAATTCGGAtgcGCTTGAGAGTACCTG	
Tac1	attcgggtaccggcgcactcccgttctggata	KpnI
Tac2	attcgggatccgggtgcctagtttcctgtgtg	BamHI

[a] Capital letters correspond to nucleotides of the MC58 genome sequence and small letters to either nucleotides of *E. coli* genome or plasmid sequence origin or nucleotides added for cloning reasons, underlined letters indicate sequences of restriction enzyme sites.

[b] Enzymes for which the restriction sites are present in the sequence of the primer, added for cloning reasons.



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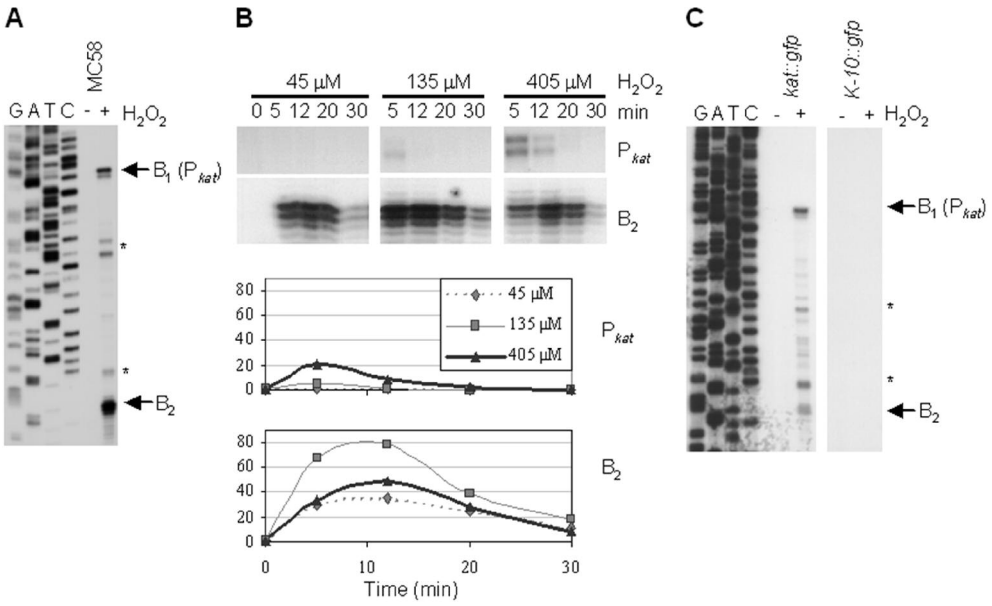

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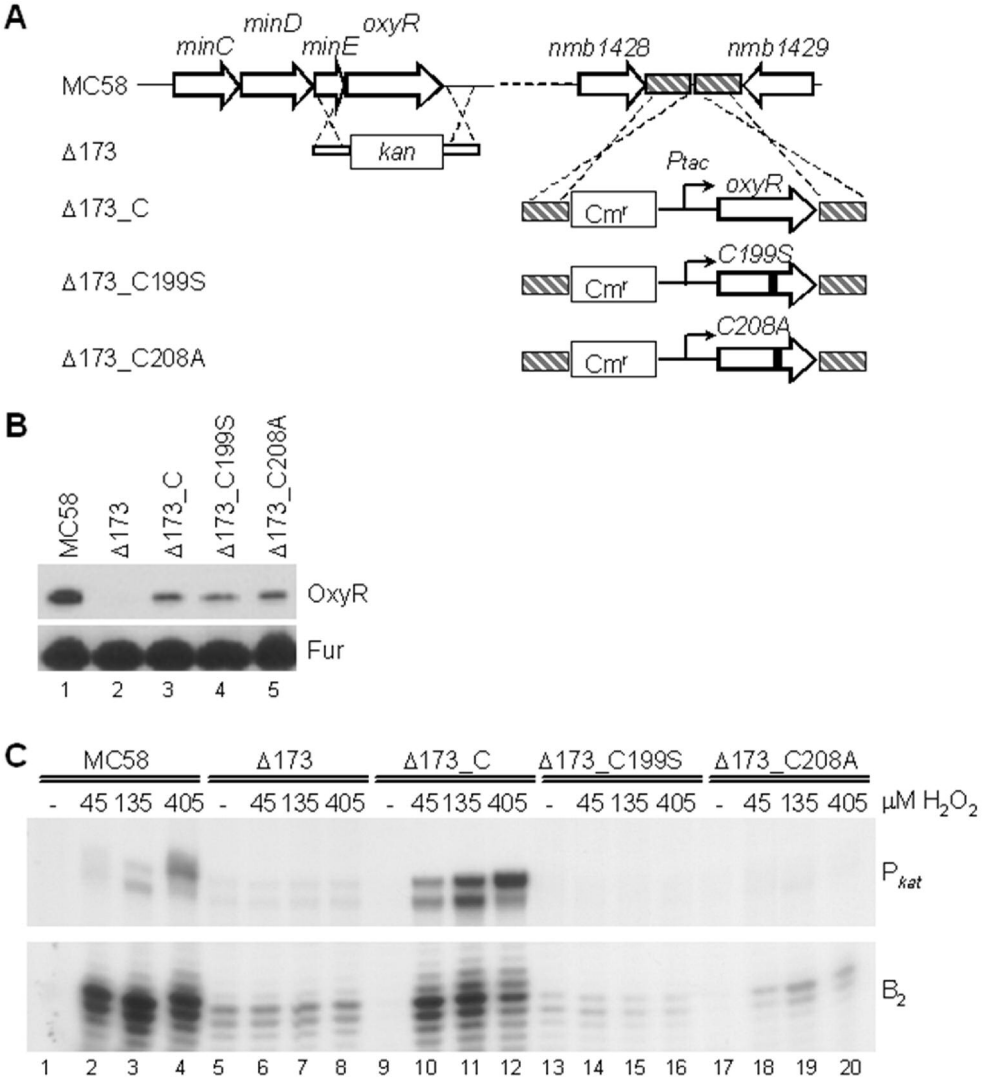

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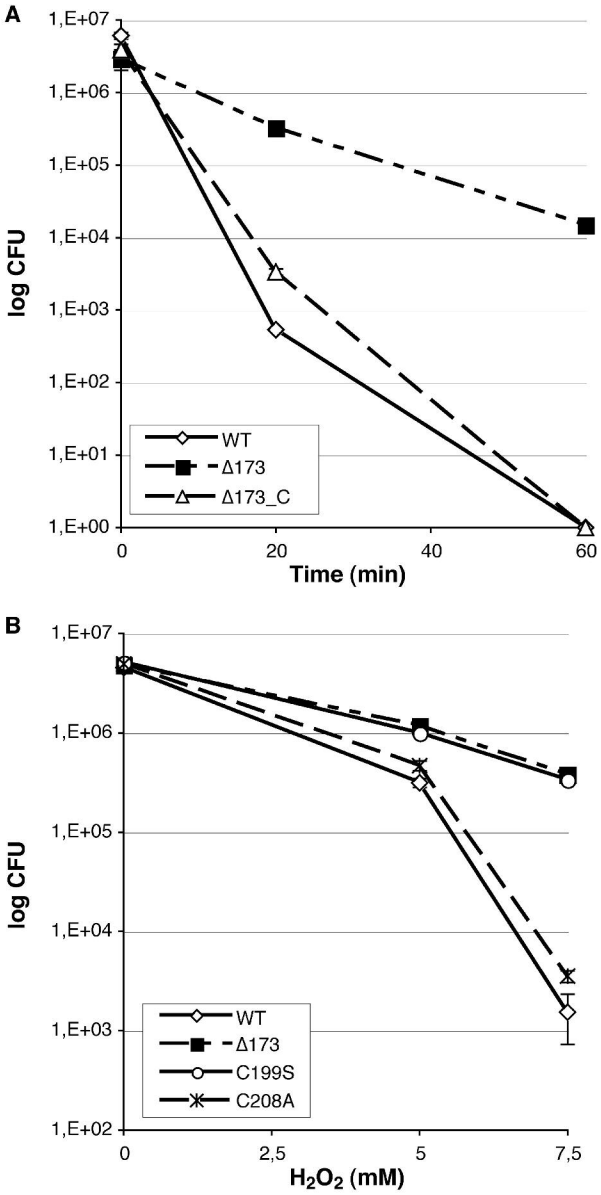

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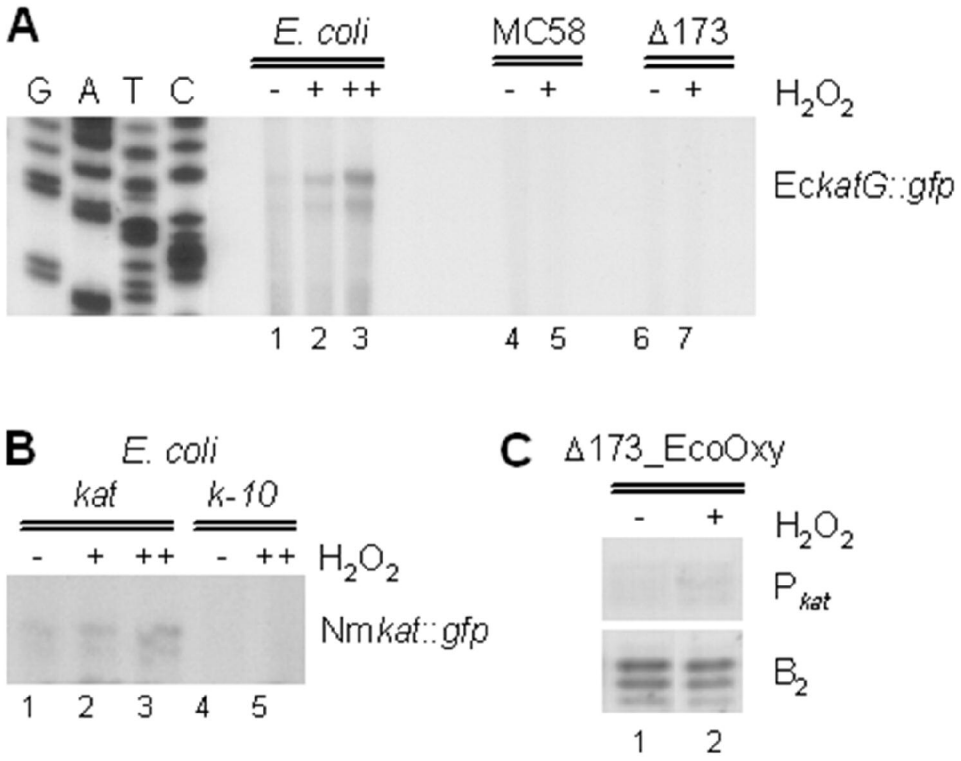

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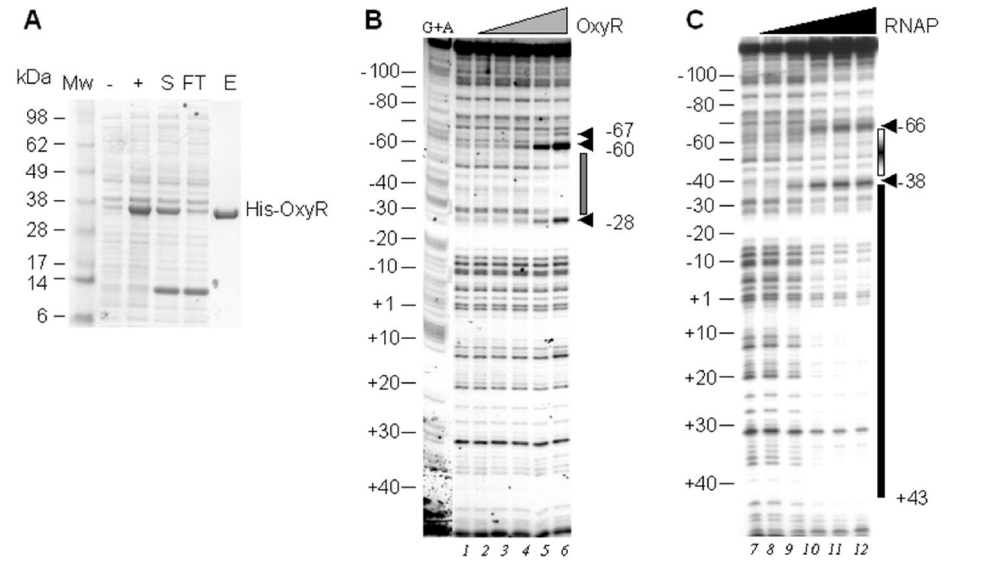

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   rrnD P1                             AGAAAAAAAGATCAAAAAAATActtgtgc
   rrnB P1                             AGAAAATTATTTTAAATTTCCTcttgtca
Nm PkatA        ACAAGAACCGATTGCTTATAATAATATTAAATCGATTTCATAGTTTTAATAGCGAAAATCTTGGCG TATAGTCGCATCC

Ec PkatG      ACAATATGTAAGATCGCAACTATCGCATCCGTGGATTAATTCAATTATAACTTCTCTCTAACGCTGTGTATCGTAACGGTA
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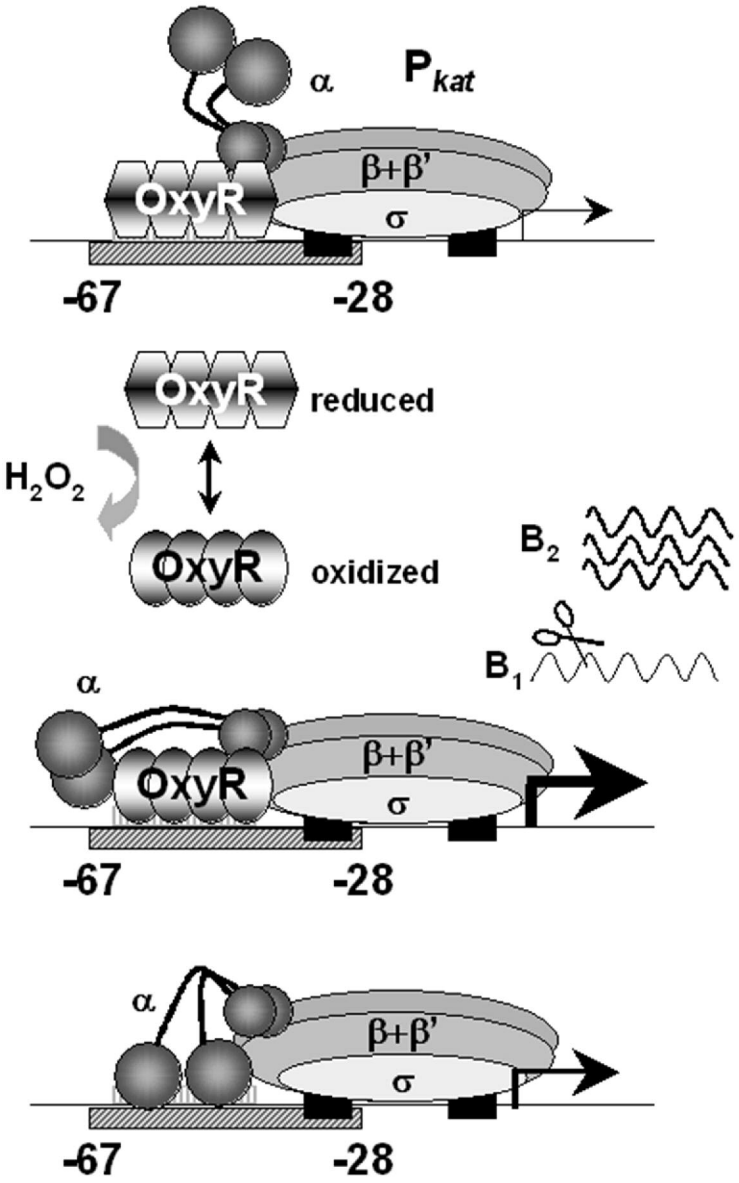

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