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OxyR Acts as a Repressor of Catalase Expression in *Neisseria gonorrhoeae*

Hsing-Ju Tseng,[1] Alastair G. McEwan,[1] Michael A. Apicella,[2] and Michael P. Jennings[1]*

Centre for Metals in Biology and Department of Microbiology and Parasitology, School of Molecular and Microbial Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia,[1] and Department of Microbiology, University of Iowa, Iowa City, Iowa 52242[2]

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It has been reported that *Neisseria gonorrhoeae* possesses a very high level of catalase activity, but the regulation of catalase expression has not been investigated extensively. In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, it has been demonstrated that OxyR is a positive regulator of hydrogen peroxide-inducible genes, including the gene encoding catalase. The *oxyR* gene from *N. gonorrhoeae* was cloned and used to complement an *E. coli oxyR* mutant, confirming its identity and function. The gene was inactivated by inserting a kanamycin resistance cassette and used to make a knockout allele on the chromosome of *N. gonorrhoeae* strain 1291. In contrast to *E. coli*, the *N. gonorrhoeae oxyR*::*kan* mutant expressed ninefold-more catalase activity and was more resistant to hydrogen peroxide killing than the wild type. These data are consistent with OxyR in *N. gonorrhoeae* acting as a repressor of catalase expression.

Neisseria gonorrhoeae is a facultative aerobe with a high iron requirement and a highly active aerobic respiratory chain. These factors suggest that this bacterium would require defense systems to respond to toxic oxygen species. Furthermore, *N. gonorrhoeae* is usually associated with inflamed urogenital tissues and activated polymorphonuclear leukocytes evolving substantial amounts of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) as part of their oxygen-dependent bactericidal mechanisms. Despite the presence of this toxic environment, *N. gonorrhoeae* can be isolated from purulent exudates laden with polymorphonuclear leukocytes (3).

Hydrogen peroxide is a potent bactericidal agent. It can react with Fe(II) to generate the hydroxyl radical ($OH^{\cdot}$), a particularly potent oxidizing agent that causes damage to DNA, lipids, and proteins (14, 40). Thus, the removal of hydrogen peroxide is a critical step in the bacterial defense against oxidative killing. *N. gonorrhoeae* possesses very high levels of catalase and peroxidase (3, 13). It has been demonstrated that the presence of catalase significantly increased the ability of *N. gonorrhoeae* to resist in vitro killing by exposure to H_2O_2 and human neutrophils (16, 44). Zheng et al. (44) identified a single source of catalase activity in *N. gonorrhoeae* extracts. The amino acid sequence of the catalase of *N. gonorrhoeae* is 83% identical to that of *H. influenzae* catalase HktE (17). It was demonstrated that catalase activities were induced, increasing threefold when subjected to 1 mM H_2O_2 (43). However, the molecular aspects of the regulation of catalase expression in *N. gonorrhoeae* were not investigated.

In contrast, in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, it is well established that catalase is part of a global system regulated by *oxyR* (5). OxyR is a positive regulator of the expression of nine hydrogen peroxide-inducible genes, including those encoding catalase (*katG*), glutathione reductase (*gor*), glutaredoxin (*grxA*), alkyl hydroperoxide reductase (*ahpCF*), OxyS (a regulatory RNA), Fur (ferric uptake regulation), and Dps (a nonspecific DNA-binding protein) (5, 11, 15, 25). Mutations in *oxyR* or *oxyR*-regulated enzymes involved in the defense against reduced-oxygen compounds result in a dramatic increase in the sensitivity of *E. coli* to H_2O_2, leading to the classic phenotype of increased levels of spontaneous mutagenesis during aerobic growth (5, 20–22). OxyR is a member of the LysR-NodD family of bacterial regulatory proteins and binds to the promoters of *oxyR*-regulated genes (4, 6, 37, 42). The oxidized, but not the reduced, form of the OxyR protein activates transcription of *oxyR*-regulated genes in vitro (36). Recent studies showed that oxidation by peroxide leads to the formation of an intramolecular disulfide bond in OxyR that triggers the activation of the transcription factor (45). OxyR activation and deactivation are consequences of the C199-C208 disulfide bond formation and reduction (45).

Recently, we demonstrated that *N. gonorrhoeae* possesses only a Fe-dependent SodB but uses Mn(II) ions to protect against superoxide killing via a Sod-independent mechanism (41). Since Mn(II) is known to quench hydrogen peroxide (35), we were also interested in determining whether it contributes to defense against this oxidant and whether it exerts any regulatory effects on catalase expression. Since *N. gonorrhoeae* is a gram-negative bacterium, the question of the role of OxyR must be raised since it might be expected to have a pattern of regulation of catalase expression similar to those of the enteric bacteria. In this paper we report the unusual phenotype of the *oxyR* mutant in *N. gonorrhoeae*. We present data indicating that the mechanism of oxidative stress gene regulation by OxyR in *N. gonorrhoeae* may be quite different from the *E. coli* paradigm.

Identification and mutagenesis of the *oxyR* gene in *N. gonorrhoeae*. Using *E. coli* OxyR as the search sequence query, we identified a potential homolog in the *N. gonorrhoeae* FA1090 genome (nucleotide positions 1791204 to 1790284 in GenBank accession number AE004969 [this study] and AF514857 from strain 1291]this study]). The deduced amino acid sequence of

* Corresponding author. Mailing address: Department of Microbiology and Parasitology, School of Molecular and Microbial Sciences, The University of Queensland, Brisbane, QLD 4072, Australia. Phone: 61 7 3365 4879. Fax: 61 7 3365 4620. E-mail: jennings@mailbox.uq.edu.au.

TABLE 1. Primers used for PCR of the *oxyR* gene

Primer	Sequence	Nucleotide positions[a]
oxyRfor	5′-CGGAGAACCGGTCATCCA-3′	1791602–1791619
oxyRrev	5′-GACGAATCTATCCATACG-3′	1790052–1790069
oxyRinfor	5′-CCGAATTGCGGTACATCG-3′	1791177–1791194
oxyRinrev	5′-CCTAGTCGTGGATAAAAC-3′	1790283–1790300
oxyRORFfor	5′-CCGGAATTCCAGAAAAAGGTATAGGAC-3′	1791205–1791222
oxyRORFrev	5′-CGCGGATTCCTAGTCGTGGATAAAACT-3′	1790284–1790301
kanfor	5′-TTATCGGCCGAAGCCACGTTGTGTCTCA-3′	
kanrev	5′-GCTGAGATCTGCCTCGTGAAGAAGG-3′	

[a] Nucleotide positions in GenBank accession number AE004969 . Note that accession number AE004969 correspond to the genome sequence of *N. gonorrhoeae* strain FA1090.

N. gonorrhoeae OxyR has 37% sequence identity and 59% sequence similarity at the protein level with *E. coli* OxyR. To determine whether this locus in *N. gonorrhoeae* was involved in gene regulation, we constructed an insertional mutation in the *oxyR* gene in *N. gonorrhoeae* strain 1291 (9). The method of construction of a knockout mutant in *N. gonorrhoeae* was described previously (41). Briefly, the *oxyR* fragment was amplified by PCR (30) using the oxyRfor and oxyRrev primers (Table 1 and Fig. 1), and a kanamycin resistance cassette (pUC4Kan; Pharmacia) was inserted into the *Eag*I site in the coding region of the gene. This plasmid was designated pHJT*oxyR*::kan-4 for the *oxyR*::*kan* mutant. This plasmid was linearized with *Eco*RI and transformed into *N. gonorrhoeae* strain 1291, and recombinant kanamycin-resistant colonies were selected. Both PCR (using the oxyRfor, oxyRrev, kanfor, and kanrev primers [Table 1 and Fig. 1]) and Southern blotting (34) using the *oxyR* gene labeled with digoxigenin (Boehringer Mannheim) as the probe showed that the *oxyR*::*kan* mutants of strain 1291 had the expected genomic organization (results not shown).

Level of catalase in the *oxyR* mutant. In *E. coli* and *S. enterica* serovar Typhimurium, OxyR is established as a positive regulator of catalase expression (5). Therefore, we expected that in *N. gonorrhoeae* the catalase level in the *oxyR*::*kan* mutant of strain 1291 would be lower than that in the wild type. It was also expected that when cells were exposed to H_2O_2, catalase would not be induced in the mutant in contrast to the wild type. The level of catalase activity present in cell extracts of bacteria grown overnight on brain heart infusion (BHI) agar containing 10% levinthal (2) and 1% supplex (Microdiagnostics) in the presence and absence of 100 μM Mn was measured by the method of Aebi (1). The reason we tested Mn in this

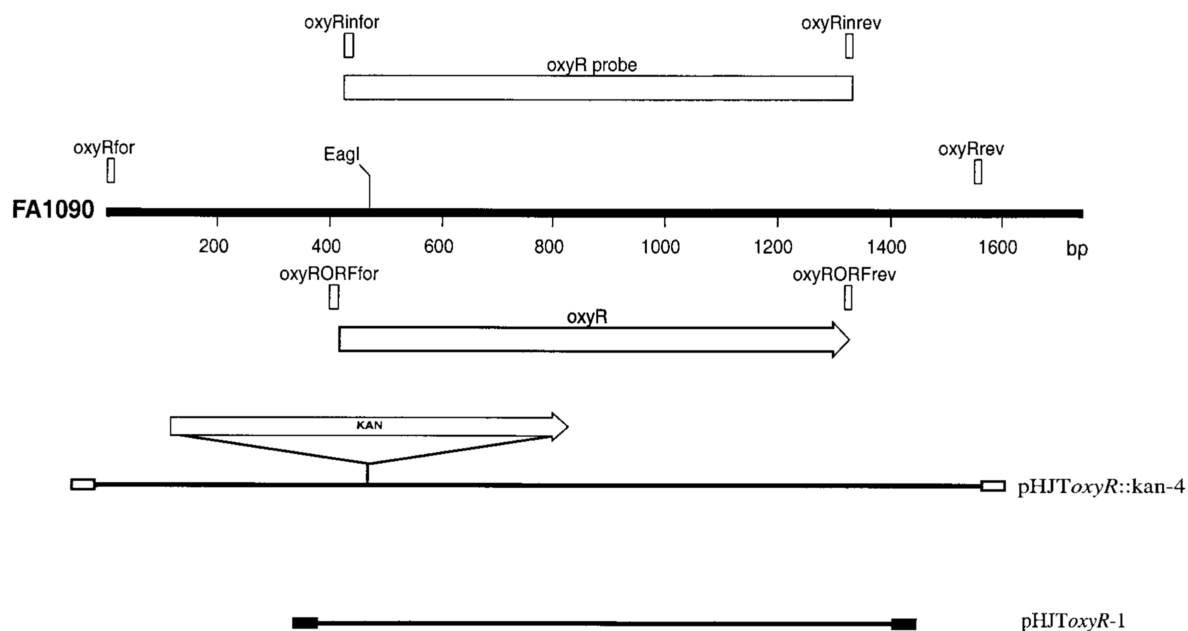

FIG. 1. Maps of *oxyR* region, probes, and plasmids. The thick black line labeled FA1090 represents restriction endonuclease map of a region of the *N. gonorrhoeae* strain FA1090 genome sequencing project (GenBank accession number AE004969). The white arrow beneath the FA1090 line indicates the orientation and location of the ORF identified in the sequence. Below the ORF, the thin black lines represent the plasmids constructed during this work. The vectors of these plasmids are represented by boxes: white for pT7Blue (Novagen) and black for pGEM T-Easy (Promega). The restriction endonuclease site shown indicates where the Kan^r cassette was inserted. Rectangular boxes above the FA1090 line represent the PCR products used as probes in this study. The *oxyR* probe was constructed from PCR products utilizing primers oxyRinfor and oxyRinrev.

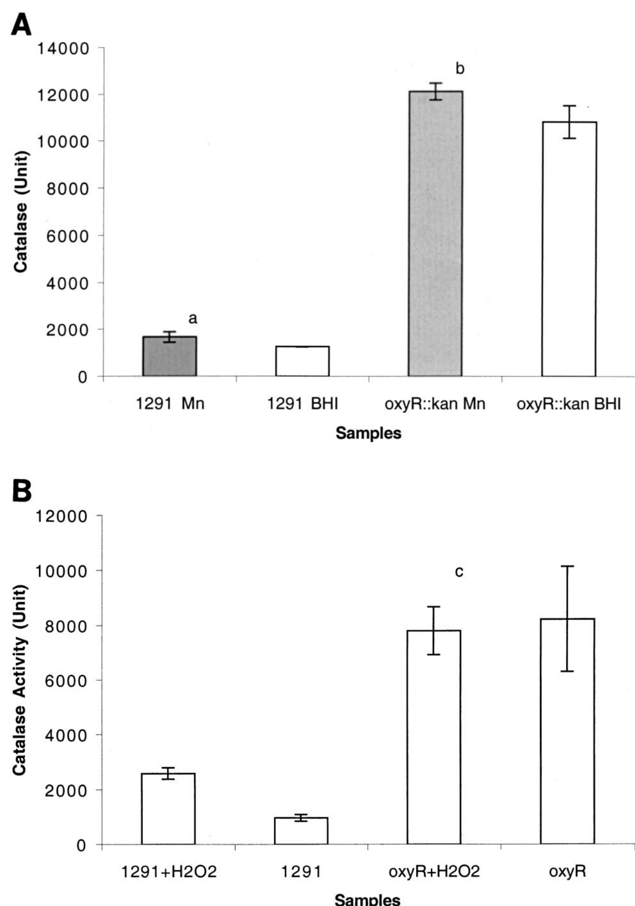

FIG. 2. Catalase activities of *N. gonorrhoeae* 1291 (wild type) and *oxyR*::*kan* mutant during growth overnight (A) and H_2O_2 treatment (B). In these assays, cells were grown on BHI agar containing 10% levinthal (2) and 1% supplex (Biomerieux) (white columns) or BHI agar containing levinthal, supplex, and 100 μM $MnSO_4$ (shaded columns). Experiments were performed in triplicate. The *y*-axis error bars each represent ±1 standard deviation of the mean. Student's *t* test was performed to determine the statistical significance between different samples. The unit of catalase is reported the concentration of H_2O_2 decomposed per minute per milligram of total protein. In the wild type, Mn increased the catalase activities slightly ($P = 0.04135$ [a]). Mn supplementation also increased the catalase activities slightly in the *oxyR*::*kan* mutant ($P = 0.06356$ [b]). H_2O_2 did not induce the catalase activity significantly in the *oxyR*::*kan* mutant ($P = 0.6417$ [c]).

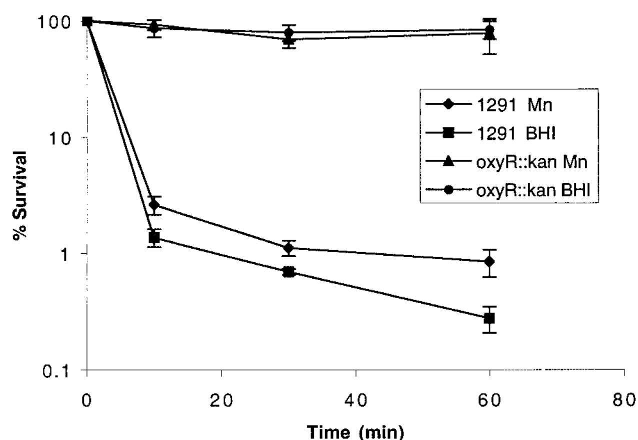

FIG. 3. H_2O_2 survival test of *N. gonorrhoeae* 1291 (wild type) and *oxyR*::*kan* mutant. In the assay, cells were grown on BHI agar containing levinthal and supplex with or without 100 μM $MnSO_4$. Experiments were performed in triplicate. The *y*-axis error bars each indicate ±1 standard deviation of the mean.

assay is because we previously demonstrated that Mn is involved in resistance to oxidative stress (41), we were interested in further investigating a potential role of Mn in modulation of catalase activity and consequent resistance to H_2O_2 killing. The decomposition of H_2O_2 can be measured directly by the decrease in absorbance at 240 nm (ε_{240} = 0.0394 $mmol^{-1}$ cm^{-1}) (26). Surprisingly, the *oxyR*::*kan* mutant of *N. gonorrhoeae* had an approximately ninefold-higher catalase activity than the wild type (strain 1291) (Fig. 2A). This suggested that OxyR in *N. gonorrhoeae* works as a repressor, which is opposite to the action of its homolog in *E. coli* and *S. enterica* serovar Typhimurium. When the cells were grown on Mn-supplemented agar media, both the wild type and the *oxyR*::*kan* mutant had slightly higher catalase activities than those cells grown on BHI agar only (Fig. 2A, $P = 0.04135$ and 0.06356, respectively).

When log-phase cells were challenged with 1 mM H_2O_2, the wild type grown on normal media showed a 2.7-fold increase in catalase activity level (Fig. 2B). However, the *oxyR*::*kan* mutant strain showed no increase in catalase activity when challenged with H_2O_2 (Fig. 2B, $P = 0.6417$).

The *oxyR* mutant is resistant to hydrogen peroxide killing. The above experiment showed that the *oxyR*::*kan* mutant of *N. gonorrhoeae* expressed much higher catalase activity than the wild type. This suggested that the *oxyR*::*kan* mutant might be more resistant to H_2O_2 than wild-type cells. Figure 3 shows that the *oxyR*::*kan* mutant was indeed more resistant to H_2O_2 than the wild-type cells, and under the conditions tested, there was only a slight loss of viability in the killing experiment. Again, this is opposite to *oxyR*-dependent regulation of catalase expression in *E. coli* and *S. enterica* serovar Typhimurium (5). Mn(II) supplementation conferred only slightly increased resistance to H_2O_2 in the wild-type cells, but not in the *oxyR*::*kan* mutant (Fig. 3).

An *oxyR* mutant is more resistant to xanthine-xanthine oxidase killing. We were also interested in determining whether the *oxyR*::*kan* mutant was also resistant to killing by oxygen radicals produced external to the cells. The xanthine/xanthine oxidase system (described previously in reference 41) produces superoxide anion and also hydrogen peroxide (43). Using this system, it was observed that the *oxyR*::*kan* mutant was significantly more resistant to killing than the wild-type cells (Fig. 4A). The results shown in Fig. 4A indicate that the significant difference in protection seen in the *oxyR*::*kan* mutant results from the very high levels of catalase activity expressed by this strain (Fig. 2). The addition of Mn resulted in increased protection in both strains, presumably due to Mn-mediated dismutation of superoxide anion (41).

Paraquat killing assays (12) were used to determine the effects of the *oxyR*::*kan* mutations of defense against intracellular superoxide anion. Figure 4B shows that the sensitivity to paraquat killing of the *oxyR*::*kan* mutant is similar to that of the

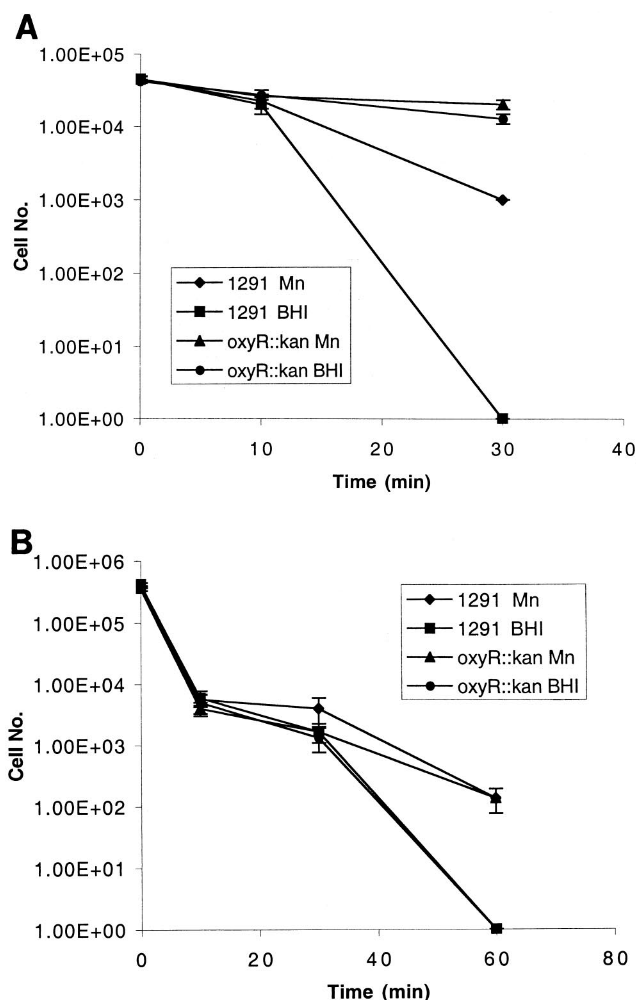

FIG. 4. (A) Xanthine-xanthine oxidase and (B) paraquat killing assays of *N. gonorrhoeae* (wild type) and *oxyR*::*kan* mutant. Cells were grown on BHI agar containing levinthal and supplex with or without 100 μM $MnSO_4$. Experiments were performed in triplicate. The *y*-axis error bars each indicate ± 1 standard deviation of the mean. 1.00E+05, 1.0×10^5.

wild type in both unsupplemented and Mn-supplemented media. This result suggests that the increased catalase levels in the *oxyR*::*kan* mutant do not provide increased protection from superoxide killing. Although in some systems the removal of hydrogen peroxide from superoxide anion dismutation could be a rate-limiting step, the difference in catalase levels between the wild-type and *oxyR*::*kan* strains was not sufficient to reveal a difference in protection in these assays.

Sequence alignment and analysis of the *oxyR* gene. A number of *oxyR* homologs have been identified and characterized in many different bacteria, such as *Mycobacterium marinum* (28), *Mycobacterium tuberculosis* (33), *Brucella abortus* (18), *Haemophilus influenzae* (24), *Xanthomonas campestris* (23), and *Bacteroides fragilis* (29). Alignment of these amino acid sequence was performed by MultAlin (http://prodes.toulouse.inra.fr/multalin/multalin.html [7]) and is shown in Fig. 5. Analysis of the amino acid sequence of OxyR of *N. gonorrhoeae* revealed that it contains all the typical features of the OxyR proteins. OxyR belongs to the LysR family which is composed of autoregulatory transcriptional regulators (LTTRs) (32). The region of greatest amino acid sequence identity between LTTRs is the 66 N-terminal residues. The central portion of this conserved region (residues 23 to 42) is nearly 40% identical in all LTTRs; secondary structure predictions (10) and other methods (8) predict that it contains a helix-turn-helix DNA-binding motif. By comparison with the LysR family, the helix-turn-helix of OxyR is from residues 19 to 37 and the DNA-binding domain is near residues 1 to 60 (Fig. 5). Furthermore, the C199 and C208 residues that form the disulfide bond when OxyR is oxidized are strongly conserved in these OxyR homologs.

Complementation of an *oxyR* mutation in *E. coli*. Unexpectedly, OxyR in *N. gonorrhoeae* acts as a repressor of gene expression, rather than an activator as seen in *E. coli* and *S. enterica* serovar Typhimurium. The ability of the *N. gonorrhoeae oxyR* gene to complement the *E. coli oxyR* mutant was therefore investigated. The *oxyR* open reading frame (ORF) of *N. gonorrhoeae* 1291 was amplified by PCR using oxyRORFfor and oxyRORFrev primers (Table 1) and cloned into pGEM-T Easy (Promega) (Fig. 1). This construct, designated pHJT*oxyR*-1, and the vector control (pGEM-T Easy vector only) were then transformed to *E. coli* K-12 and GS09 (*oxyR*::kan mutant) kindly provided by Gisela Storz (National Institutes of Health) to see if the *oxyR* gene of *N. gonorrhoeae* could complement the defect in the *E. coli* GS09 mutant. Multiple independent *E. coli* clones were obtained and analyzed via a H_2O_2 disk assay by a modified version of the method of Christman et al. (5). In this assay, *E. coli* cells were grown in Luria-Bertani (LB) broth at 37°C with shaking overnight. Portions (100 μl) of the cell cultures grown overnight were added to LB top agar and spread onto LB agar. Then, 10 μl of 3% H_2O_2 (Riedel-de Haën) was pipetted onto 3MM Whatman paper disks (0.5-cm diameter), and these disks were placed on top agar and incubated at 37°C overnight. The assay was repeated on three separate occasions. The result showed that *E. coli* GS09 was more sensitive to H_2O_2 killing than the wild type (Fig. 6A and B), as shown by a larger zone of growth inhibition. However, it was demonstrated that the *oxyR* gene from *N. gonorrhoeae* could complement the defect in the *E. coli* GS09 mutant (Fig. 6C and D show representative results). The complemented strain was significantly more resistant to H_2O_2 killing than the *oxyR*::kan mutant (GS09) ($P = 0.01757$) and was similar to the wild type (K-12) ($P = 0.5813$) (Fig. 6D). Therefore, OxyR from *N. gonorrhoeae* does function as an activator of gene expression in *E. coli*.

A search for the OxyR-binding site of the catalase gene in *N. gonorrhoeae*. A consensus sequence for OxyR binding has been proposed and is comprised of four ATAG nucleotide elements spaced at regular intervals (27, 39). The 287-bp region between the *kat* gene-coding sequence and the adjacent gene (RNA polymerase sigma factor which lies upstream of and is transcribed in the direction opposite that of the *kat* gene) were examined in *N. gonorrhoeae* strains FA1090 (nucleotide positions 1730489 to 1730774 in GenBank accession number AE004969), 2821 (nucleotide positions 175 to 460 in GenBank accession number U35457 [17]), and 1291 (identical to FA1090 [this study]). No obvious binding sites were observed (data not shown).

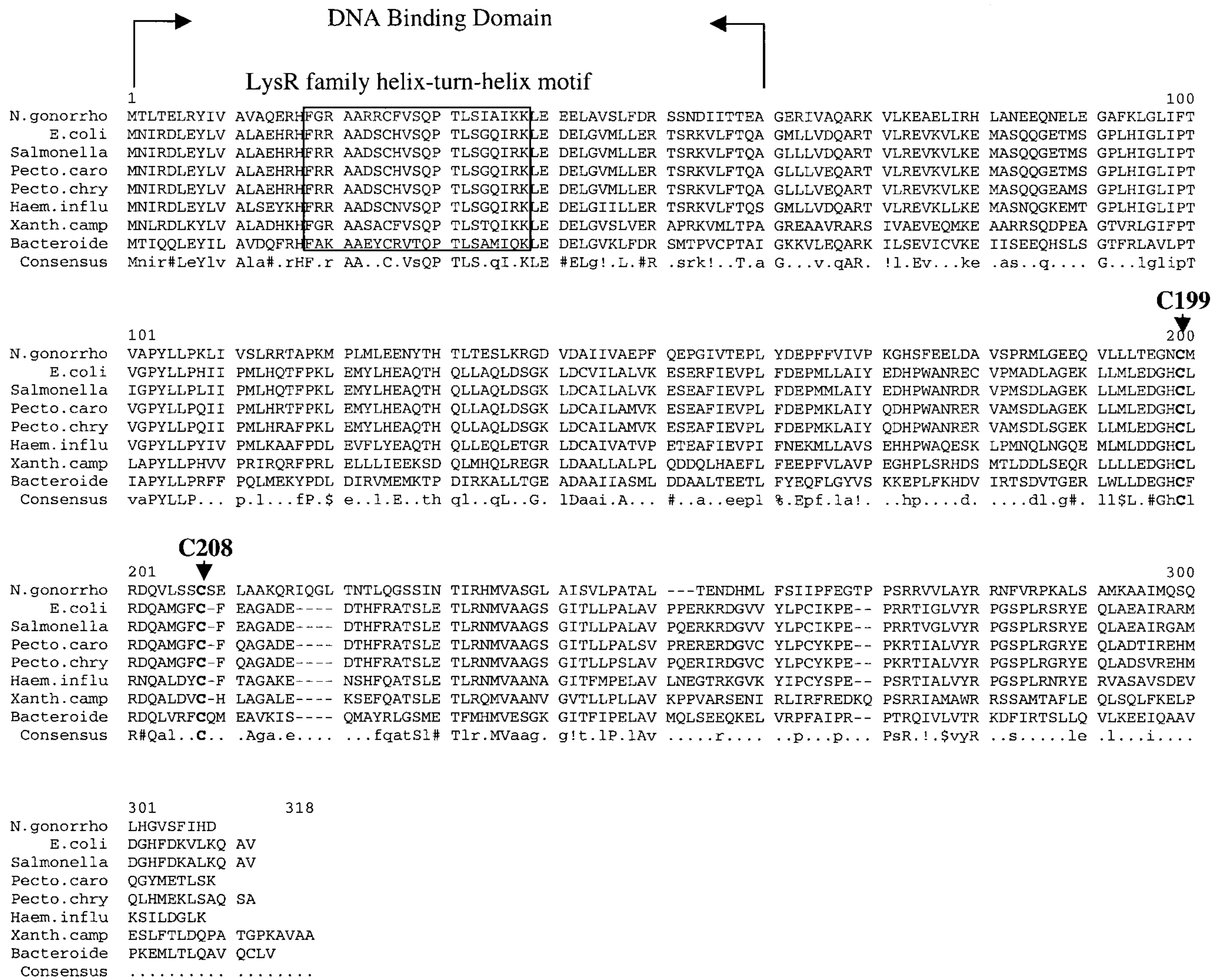

FIG. 5. Sequence comparison of OxyR homologs from different organisms. *N. gonorrhoeae* (N. gonorrho) (GenBank accession number AF514857), *E. coli* (GenBank accession number NP418396), *Salmonella enterica* (GenBank accession number NP457935), *Pectobacterium carotovorum* (Pecto.caro) (GenBank accession number AAC72242), *Pectobacterium chrysanthemi* (Pecto.chry) (GenBank accession number CAB40388), *Haemophilus influenzae* (Haem.influ) (GenBank accession number NP438728), *Xanthomonas campestris* (Xanth.camp) (GenBank accession number AAC45427), and *Bacteroides fragilis* (Bacteriode) (GenBank accession number AAG02619) sequences were used. The DNA-binding domain is shown between the two arrows, and the helix-turn-helix motif is boxed. Gaps introduced to maximize alignment are indicated by hyphens. The conserved residues are shown in the consensus sequence below the sequences. The lowercase letters in the consensus sequence indicate a low consensus residue while the periods indicate no consensus. Symbols: !, I or V; $, L or M; %, F or Y; #, N, D, Q, E, B, or Z.

Discussion. OxyR is a member of the LysR family of transcriptional regulators. Like the other LysR regulators, OxyR contains an N-terminal DNA-binding region and a central region associated with signaling (32). In the case of *E. coli* OxyR, peroxide is sensed via two cysteine residues that form an intramolecular disulfide bridge via a reaction with hydrogen peroxide. All of the available evidence indicates that, like most LysR regulators, OxyR in enteric bacteria acts as a positive regulator of gene expression. In contrast, our data suggest that in *N. gonorrhoeae* this transcription factor acts as a repressor. This could be due to a fundamental difference in OxyR between *N. gonorrhoeae* and *E. coli* or differences in the promoters of *kat* genes in these organisms. We have shown that the *oxyR* gene of *N. gonorrhoeae* has sequence features similar to those of *E. coli*. The helix-turn-helix DNA-binding motif in *N. gonorrhoeae* and *E. coli* are conserved (63% identity), and the *oxyR* gene of *N. gonorrhoeae* also contains the two conserved cysteine residues, C199 and C208 (Fig. 5). Furthermore, we showed that the *oxyR* gene of *N. gonorrhoeae* can complement the *E. coli oxyR*::*kan* mutant, demonstrating that OxyR of *N. gonorrhoeae* acts as an activator in *E. coli*. However, our data are consistent with OxyR in *N. gonorrhoeae* acting as a repressor by binding directly to the promoter of the catalase gene in its reduced form and being released upon oxidation by hydrogen peroxide. Alternately, OxyR could have an indirect role, acting via a regulatory cascade on the catalase promoter. The OxyR protein of *E. coli* specifically recognizes regions of DNA that share very little sequence similarity, and its func-

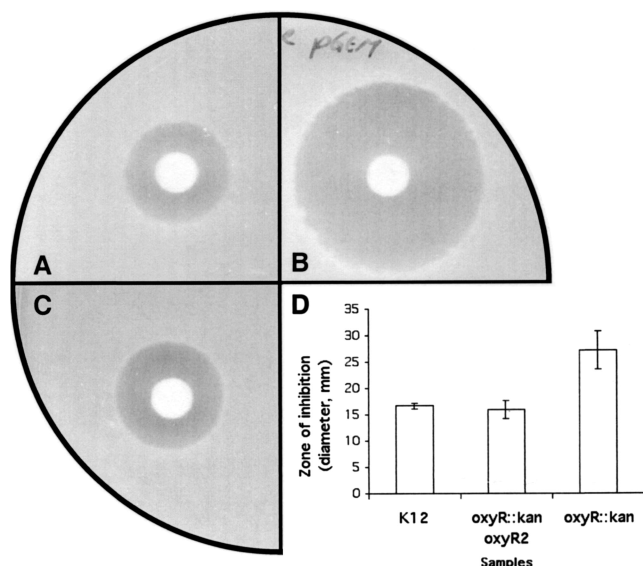

FIG. 6. H_2O_2 disk assay. Photos showing the zones of inhibition by H_2O_2 in *E. coli* K-12 (wild type) (A), GS09 (*oxyR*::*kan* mutant) (B), and *E. coli* K-12 strain GS09 complemented with the *oxyR* gene (pHJT*oxyR*-1) from *N. gonorrhoeae* (C). (D) Histogram showing the results of H_2O_2 disk assay in *E. coli*. In the assay, cells were grown on LB agar in the presence or absence of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Sensitivity to H_2O_2 was measured by the zone of inhibition (diameter). The zone of inhibition was measured in three dimensions, and the mean values and standard deviations were calculated. The *y*-axis error bars each indicate ±1 standard deviation of the mean. Student's *t* test was performed to determine the statistical significance among different samples. The *E. coli* K-12 *oxyR*::*kan* mutant was significantly more sensitive to H_2O_2 than the wild type and the complemented strain (P = 0.03315 and 0.01757, respectively).

tional binding sites are unusually large (>45 bp) (6, 38). Despite this, the alignment of the OxyR-regulated promoters indicates four putative OxyR-binding tetranucleotide sequences upstream of the −35 promoter elements (39). Toledano et al. (39) found that the two forms of OxyR in *E. coli* make different DNA contacts: oxidized OxyR recognizes four ATAG nucleotide elements in four adjacent major grooves, while reduced OxyR recognizes ATAG nucleotide elements present in two pairs of adjacent major grooves separated by one helical turn. Our examination of the catalase gene in *N. gonorrhoeae* strains 1291 and FA1090 revealed no obvious OxyR-binding site. This is consistent with the finding of Johnson et al. (17), who reported that unlike *hktE*, the gonococcal *kat* gene was not preceded by any sequence that exhibited significant homology with the OxyR-binding consensus sequence. In *H. influenzae*, an *oxyR* mutant was more sensitive to killing by H_2O_2 than the wild type (24). Again, it had a phenotype similar to those of *E. coli* and *S. enterica* serovar Typhimurium (5, 20–22) but different from that of *N. gonorrhoeae* (Fig. 3).

In *E. coli*, it is known that although expression of peroxide defense genes is activated by OxyR in the exponential phase, in the stationary phase, expression of these genes is controlled by the starvation-induced sigma factor RpoS (31). In *N. gonorrhoeae*, it has been established that the *rpoS* gene is not active (19). Thus, the regulation of peroxide defense response in this bacterium via repression by OxyR represents a much simpler peroxide defense system than that found in enteric bacteria. Analysis and comparisons of the organization of *oxyR* genes from several different microorganisms revealed that they are usually linked to antioxidant or associated genes. For instance, the *ahpC* (alkyl hydroperoxide reductase) gene in *Mycobacterium leprae* and *M. tuberculosis* (28) is upstream of the *oxyR* gene and is divergently transcribed from *oxyR*. In *Pseudomonas aeruginosa*, *recG* (DNA repair enzyme) is located downstream of *oxyR* (27). The *oxyR* gene in *N. gonorrhoeae*, however, is flanked by *minD* (septum site-determining protein) and outer membrane protein I, which do not have obvious roles in oxidative stress.

Here we report that catalase is induced by H_2O_2 via OxyR-mediated derepression. This unique arrangement is more interesting when we consider that although *N. gonorrhoeae* is known for very high levels of catalase production, the catalase activity of the *oxyR*::*kan* mutant is fourfold higher than that of the maximally induced wild-type strain. Furthermore, the *oxyR*::*kan* mutant is far more resistant to H_2O_2 killing than the wild-type strain, presumably due to the increased catalase activity. The reason why a regulatory system has evolved in *N. gonorrhoeae* that responds to H_2O_2 via OxyR but does not derepress the promoter to allow maximal expression, effectively limiting catalase activity, remains to be resolved.

Nucleotide sequence accession number. The GenBank accession number for the completed *N. gonorrhoeae* genome is AE004969.

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