Characterization of OxyR as a Negative Transcriptional Regulator That Represses Catalase Production in Corynebacterium diphtheriae

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Abstract

*Corynebacterium* diphtheriae and *Corynebacterium glutamicum* each have one gene (*cat*) encoding catalase. In-frame Δ*cat* mutants of *C. diphtheriae* and *C. glutamicum* were hyper-sensitive to growth inhibition and killing by H$_2$O$_2$. In *C. diphtheriae* C7(b), both catalase activity and *cat* transcription decreased ~2-fold during transition from exponential growth to early stationary phase. Prototypic OxyR in *Escherichia coli* senses oxidative stress and it activates katG transcription and catalase production in response to H$_2$O$_2$. In contrast, exposure of *C. diphtheriae* C7(b) to H$_2$O$_2$ did not stimulate transcription of *cat*. OxyR from *C. diphtheriae* and *C. glutamicum* have 52% similarity with *E. coli* OxyR and contain homologs of the two cysteine residues involved in H$_2$O$_2$ sensing by *E. coli* OxyR. In-frame ΔoxyR deletion mutants of *C. diphtheriae* C7(b), *C. diphtheriae* NCTC13129, and *C. glutamicum* were much more resistant than their parental wild type strains to growth inhibition by H$_2$O$_2$. In the *C. diphtheriae* C7(b) ΔoxyR mutant, *cat* transcripts were about 8-fold more abundant and catalase activity was about 20-fold greater than in the C7(b) wild type strain. The oxyR gene from *C. diphtheriae* or *C. glutamicum*, but not from *E. coli*, complemented the defect in ΔoxyR mutants of *C. diphtheriae* and *C. glutamicum* and decreased their H$_2$O$_2$ resistance to the level of their parental strains. Gel-mobility shift, DNaseI footprint, and primer extension assays showed that purified OxyR from *C. diphtheriae* C7(b) bound, in the presence or absence of DTT, to a sequence in the *cat* promoter region that extends from nucleotide position −55 to −10 with respect to the +1 nucleotide in the *cat* ORF. These results demonstrate that OxyR from *C. diphtheriae* or *C. glutamicum* functions as a transcriptional repressor of the *cat* gene by a mechanism that is independent of oxidative stress induced by H$_2$O$_2$.

Introduction

Acrobic organisms have specific mechanisms to protect themselves from reactive oxygen species (ROS) such as superoxide radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), which can be generated by incomplete reduction of O$_2$ during respiration. The Fenton reaction, which generates hydroxyl radicals by reduction and oxidation of Fe ions in the presence of ROS, has been proposed as a mechanism for cell damage [1]. The ROS may cause oxidative damage to DNA, protein, and lipid [2]. Major defense systems against ROS often involve superoxide dismutase (SOD), catalase, and peroxidase, which function cooperatively to convert the ROS into O$_2$ and H$_2$O.

*Escherichia coli* produces two types of catalase, both of which can detoxify H$_2$O$_2$ by converting it to H$_2$O and O$_2$. The first is a bifunctional catalase hydroperoxidase I (HPI) encoded by katG that has both catalase and peroxidase activities, and the katG gene is transcriptionally activated by the positive regulator OxyR [3]. A monofunctional HPII encoded by katE has catalase activity only, and katE is activated during stationary phase [3] but is not induced by OxyR [4].

The two best characterized transcriptional regulators that mediate responses to oxidative stress in *E. coli* are SoxRS and OxyR. SoxR is a transcriptional activator with an iron-sulfur cluster that can be oxidized to the [2Fe-2S] state by exposure to the superoxide radical or nitric oxide. Both the reduced and the oxidized forms of SoxR can bind to its DNA target sequence [5,6], but only the oxidized form of SoxR increases the transcription of *soxS*. The SoxS protein then activates transcription of *sodA* (superoxide dismutase), *zuf* (glucose-6-phosphate dehydrogenase), *fpr* (NADPH-ferredoxin reductase), *nfo* (DNA repair endonuclease IV), and *accAB* (an efflux pump) in *E. coli* [7,8].

The other major redox-sensing protein from *E. coli*, OxyR, functions both as a hydrogen peroxide sensor and as a transcriptional activator. *E. coli* OxyR, a member of the LysR family of transcription factors [9], has a helix-turn-helix (HTH) DNA binding motif in its N-terminal region and regulatory and oligomerization motifs in its C-terminal region. Oxidation of OxyR by hydrogen peroxide converts two cysteine residues (Cys199 and Cys208) to a disulfide bond, and the oxidized form of OxyR can then bind to specific DNA sequences in the regulatory regions of target genes [10]. The OxyR-activated genes...
in E. coli include dps (a DNA-binding protein from starved cells), gorA (GSH reductase), gxhA (glutaredoxin), katG (catalase/peroxidase), ahpCF (alkyl hydroperoxide-NADPH oxidoreductase), and fur (an iron-binding repressor of iron transport) [7,11,12]. Products of genes in the OxyR regulon have many important functions in antioxidant defenses, including degradation of hydrogen peroxide by catalase and protection of DNA from oxidative attack by Dps proteins. In addition, E. coli OxyR activates production of OxyS, a small protein that regulates as many as 20 additional gene products [13].

Recent studies in Neisseria gonorrhoeae and Neisseria meningitidis indicate that OxyR can function both as a repressor of catalase gene expression in the absence of oxidative stress and as an activator of catalase gene expression in response to H2O2. In an oxyR mutant of N. gonorrhoeae, the basal level of catalase activity was greatly increased, and induction of catalase by hydrogen peroxide was abolished [14,15]. In wild-type N. meningitidis, transcription of the catalase gene was activated in response to H2O2 in an OxyR-dependent manner, but transcription of the catalase gene in an oxyR null mutant was at a constitutive intermediate level between the uninduced and H2O2-induced levels in the wild type strain [16].

Corynebacterium diphtheriae is gram-positive, non-spore-forming aerobic bacterium that produces diphtheria toxin and causes the severe respiratory disease diphtheria [17]. The diphtheria toxin repressor (DtxR) in C. diphtheriae represses diphtheria toxin production and iron uptake in response to high-iron conditions [10,19,20]. A C. diphtheriae C7(β) ΔdexR mutant exhibited increased susceptibility to growth inhibition and killing in response to oxidative stress [21]. Only a few studies other studies of oxidative stress responses have been reported in bacteria from the genus Corynebacterium. Merkamm and Guyonvarch cloned the sodA gene from C. menenassa and reported that superoxide dismutase played a role in cell viability [22]. The sodA gene was also cloned from C. glutamicum and shown to encode a strictly manganese-dependent form of superoxide dismutase [23].

In this study, we investigated the roles of the cat and oxyR genes of C. diphtheriae and C. glutamicum in the responses of these bacteria to H2O2-induced oxidative stress. We also characterized the effects of growth conditions on cat expression and showed that OxyR in C. diphtheriae functions as a repressor, but not as an activator, of cat by a mechanism that is independent of H2O2-induced oxidative stress.

**Methods**

**Bacterial stains, plasmids, and growth conditions**

The bacterial strains used in this study are listed in Table 1, and the plasmids used in this study are listed in Table 2. Strains of C. diphtheriae and C. glutamicum were grown at 37°C and 30°C, respectively, in heart infusion broth (Difco, Detroit, Mich.) containing 0.2% Tween 80 (designated HIBTW) or in PGT medium [24] as described for specific experiments. Low-iron PGT medium was deferrated by treatment with Chelex resin (Bio-Rad, Hercules, CA) [25], and high-iron PGT medium was made by adding 10 μM ferric chloride to the low-iron PGT medium. Exponential phase cultures of C. diphtheriae were typically harvested when A600 was between 0.8 and 1.0, and early stationary phase cultures of C. diphtheriae were typically harvested when A600 was between 6 and 7. Transconjugants of C. diphtheriae and C. glutamicum were selected on HIBTW agar plates containing 50 μg/ml of kanamycin and 30 μg/ml of nalidixic acid [21]. Strains of E. coli were grown at 37°C in Luria-Bertani (LB) medium [26] to which ampicillin (Ap), chloramphenicol (Cm), kanamycin (Km), or tetracycline (Tc) was added at a final concentration of 50, 20, 25, or 10 μg/ml, respectively, when indicated. Growth of C. diphtheriae or C. glutamicum was measured by determining A600 for culture samples appropriately diluted in 1× PBS (phosphate buffered saline) [26].

| Strains | Relevant characteristics | Reference or source |
|---------|-------------------------|---------------------|
| **Corynebacterium** | | |
| C. diphtheriae C7(β) | wild-type reference strain, tox+, lysogenic for phase β | [24] |
| C. diphtheriae | NCTC13129, wild-type strain | lab strain |
| C. glutamicum | ATCC13032, wild-type strain | lab strain |
| C7(β) Δcat | C. diphtheriae C7(β), Δcat | This study |
| C7(β) ΔoxyR | C. diphtheriae C7(β), ΔoxyR | This study |
| NCTC Δcat | C. diphtheriae NCTC13129, Δcat | This study |
| NCTC ΔoxyR | C. diphtheriae NCTC13129, ΔoxyR | This study |
| Cg Δcat | C. glutamicum ATCC13032, Δcat | This study |
| Cg ΔoxyR | C. glutamicum ATCC13032, ΔoxyR | This study |
| **E.coli** | | |
| DH5α | supE44 ΔlacU169 (Δ80 lacZ ΔM15) hsdR17 recA1 | [50] |
| endA1 gyrA96 thi-1 relA1 | |
| S17-1 | C600-REP2 2-[(Tc::Mul)(Km::Tn7) thi pro hsdR hsdM* recA | [28] |
| GC4468 | ΔlacU169 rpsL | [41] |
| JL102 | GC4468, oxyR mutant; Km | [41] |
| Origami®(DE3) | E. coli K12 F- ompT hsdSB( rB- mB-) gal dcm lacY1 aphC | Novagen |
| pLysS | (DE3) gos522-Tn10 trxB pLysS; Cm, Km, Tc | |

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Construction of in-frame deletion mutations in C. diphtheriae and C. glutamicum

(i) Construction of ΔoxyR allele for C. diphtheriae C7(b). A 789-bp upstream DNA fragment extending from nucleotide position −683 to 104 relative to the oxyR initiation codon was amplified from C. diphtheriae C7(b) genomic DNA by PCR using the forward primer oxyR-F1 (5′-CCGCGATCTCGAGTAACAG-3′, with the underlined mutation to introduce the PstI site shown in bold font) and the reverse primer oxyR-R1 (5′-GAAATCGAAGCTTGGCAGG-3′, containing the HindIII site indicated in bold font). A

| Table 2. Plasmids used in this study. |
|---------------------------------------|
| Plasmids | Relevant characteristics | Reference or source |
| pCR2.1 TOPO | pUC ori, cloning for PCR DNA fragment; Ap<sup>+</sup> | Invitrogen |
| pET-22b (+) | ori pBR322, C-terminal His<sub>6</sub>-Taq fusion vector; Ap<sup>+</sup> | Novagen |
| pET-Eco OxyR | pET-22b (+)+0.9-kb DNA containing oxyR of GC4468; Ap<sup>+</sup> | This study |
| pET-OxyR | pET-22b (+)+0.9-kb DNA containing oxyR of C7(b); Ap<sup>+</sup> | This study |
| pK19mobacsB | ori T of RP4 sacB; Km<sup>+</sup> | [27] |
| pK-ΔoxyR | pK19mobacsB+1.6-kb DNA containing in-frame deleted oxyR of C7(b); Km<sup>+</sup> | This study |
| pK-Δoxy RNCTC | pK19mobacsB+2.0-kb DNA containing in-frame deleted oxyR of NCTC13129; Km<sup>+</sup> | This study |
| pK-Δcat RNCTC | pK19mobacsB+2.0-kb DNA containing in-frame deleted cat of NCTC13129; Km<sup>+</sup> | This study |
| pK-Δoxy RCg | pK19mobacsB+2.0-kb DNA containing in-frame deleted oxyR of C. glutamicum ATCC13032; Km<sup>+</sup> | This study |
| pK-Δcat CG | pK19mobacsB+2.0-kb DNA containing in-frame deleted cat of C. glutamicum ATCC13032; Km<sup>+</sup> | This study |
| pK-PIM | ori E of pUC19, ori T of RP4, attB attP phase III, site-specific integrated plasmid; Km<sup>+</sup> | [30] |
| pK-PIM-oxyR C7(b) | pK-PIM+2.0-kb DNA containing oxyR of C7(b); Km<sup>+</sup> | This study |
| pK-PIM-oxyR Eco | pK-PIM+1.1-kb DNA containing oxyR of GC4468; Km<sup>+</sup> | This study |
| pK-PIM-oxyR Eco2 | pK-PIM+0.5-kb oxyR DNA of C7(b)+0.9-kb oxyR ORF of GC4468; Km<sup>+</sup> | This study |
| pSPZ | promoterless lacZ in shuttle vector for C. diphtheriae, pJKS1; Sp<sup>+</sup> | [31] |
| pKPL | pK-PIM with promoterless lacZ; Km<sup>+</sup> | This study |
| pPL | pK-PIM+2 lacZ fusion; Sm<sup>+</sup>/Sp<sup>+</sup>, Km<sup>+</sup> | This study |
| pPL-cat100 | pK-PIM+cat::lacZ fusion; 100-bp fragment of cat start codon; Sm<sup>+</sup>/Sp<sup>+</sup>, Km<sup>+</sup> | This study |
| pPL-cat200 | pK-PIM+cat::lacZ fusion; 200-bp fragment of cat start codon; Sm<sup>+</sup>/Sp<sup>+</sup>, Km<sup>+</sup> | This study |
| pPL-cat300 | pK-PIM+cat::lacZ fusion; 300-bp fragment of cat start codon; Sm<sup>+</sup>/Sp<sup>+</sup>, Km<sup>+</sup> | This study |
| pRK415 | ori IncP Mob RP4 lacZ<sub>C</sub>; Tc<sup>+</sup> | [42] |
| pRK-oxR Eco | pRK415+1.1-kb DNA containing oxyR of GC4468; Tc<sup>+</sup> | This study |
| pRK-oxR Eco2 | pRK415+0.5-kb oxyR DNA of C7(b)+0.9-kb oxyR ORF of GC4468; Tc<sup>+</sup> | This study |
| pT-cat A | pCR2.1 TOTO+110-bp PCR DNA fragment with cat-F12+cat-R9 primers; Ap<sup>+</sup> | This study |
| pT-cat B | pCR2.1 TOTO+110-bp PCR DNA fragment with cat-F13+cat-R10 primers; Ap<sup>+</sup> | This study |
| pT-cat C | pCR2.1 TOTO+110-bp PCR DNA fragment with cat-F14+cat-R11 primers; Ap<sup>+</sup> | This study |
| pT-cat foot | pCR2.1 TOTO+244-bp PCR DNA fragment with cat-F13+cat-R12 primers; Ap<sup>+</sup> | This study |
OxyR Represses Cat Transcription in C. diphtheriae

(i) Construction of Δcat allele for C. diphtheriae C7(β). A 1050 bp upstream DNA fragment extending from nucleotide position −1072 to +33 relative to the cat initiation codon was PCR amplified from C. diphtheriae C7(β) genomic DNA by PCR using the forward primer cat-F3 (5′-GGTCGAGAAAGCTTTACCTC-3′), with the underlined mutations to introduce the HindIII site shown in bold font) and the reverse primer cat-R2 (5′-CCCTCAGCTTGTACGGAA-3′, with the underlined mutations to introduce the XbaI site shown in bold font). The 789-bp PstI/HindIII-cut upstream DNA fragment and 969-bp HindIII/XbaI-cut downstream DNA fragment were cloned into PstI/XbaI-cut pK19mobsacB [27] to generate pK-ΔoxyRcat.

(ii) Construction of Δcat allele for C. glutamicum ATCC13032. A 1036-bp upstream DNA fragment extending from nucleotide position −964 to +72 relative to the cat initiation codon was PCR amplified from C. glutamicum genomic DNA by PCR using the forward primer cg-cat-F1 (5′-GGTCCATTGTGTCAGGAA-3′, with the underlined mutations to introduce the PstI site shown in bold font) and cg-cat-R1 (5′-GGTTTCGAGAAAGCTTTACCTC-3′) containing the HindIII site shown in bold font). A 1026-bp downstream flanking region extending from −34 to +992 relative to the cat stop codon was PCR amplified using the forward primer cg-cat-F2 (5′-GGTCGAGAAAGCTTTACCTC-3′, with the underlined mutations to introduce the HindIII site shown in bold font) and cg-cat-R2 (5′-CCCTCAGCTTGTACGGAA-3′, with the underlined mutations to introduce the XbaI site shown in bold font). The 1036-bp PstI/HindIII-cut upstream DNA fragment and the 1026-bp HindIII/XbaI-cut downstream DNA fragment were cloned into PstI/XbaI-cut pK19mobsacB [27] to generate pK-ΔoxyRcat.

(iii) Construction of ΔoxyR allele for C. diphtheriae NCTC13129. The oxyR-F1 oxyR-R1 and oxyR-F2 oxyR-R2 primer pairs described previously were used with C. diphtheriae NCTC13129 genomic DNA to PCR amplify 789-bp upstream and 869-bp downstream DNA sequences for the oxyR gene. The PstI/HindIII-cut upstream DNA fragment and HindIII/XbaI-cut downstream DNA fragment were cloned into PstI/XbaI-cut pK19mobsacB [27] to generate pK-ΔoxyRNC7TC.

(iv) Construction of Δcat allele for C. diphtheriae NCTC13129. The cat-F3/cat-R1 and cat-F2/cat-R2 primer pairs described previously were used with C. diphtheriae NCTC13129 genomic DNA to PCR amplify 1043-bp upstream and 922-bp downstream DNA sequences for the cat gene. The PstI/HindIII-cut upstream DNA fragment and HindIII/XbaI-cut downstream DNA fragment were cloned into PstI/XbaI-cut pK19mobsacB [27] to generate pK-ΔoxyRNC7TC.

(v) Construction of ΔoxyR allele for C. glutamicum ATCC13032. A 1015-bp DNA upstream DNA fragment extending from nucleotide position −974 to +41 relative to the oxyR initiation codon was amplified from C. glutamicum genomic DNA by PCR using the forward primer cg-oxyR-F1 (5′-GGTCGAGAAAGCTTTACCTC-3′, with the underlined mutations to introduce the HindIII site shown in bold font) and the reverse primer cg-oxyR-R1 (5′-GGTCGAGAAAGCTTTACCTC-3′ containing the underlined mutations to introduce the XbaI site indicated in bold font). A 961-bp DNA downstream DNA fragment extending from −25 to +936 relative to the oxyR stop codon was amplified with the forward primer cg-oxyR-F2 (5′-GGTCGAGAAAGCTTTACCTC-3′, containing the underlined mutations to introduce the XbaI site shown in bold font) and the reverse primer cg-oxyR-R2 (5′-GGTCGAGAAAGCTTTACCTC-3′, containing the EcorI site shown in bold font). The 1014-bp HindIII/XbaI-cut upstream DNA fragment and 960-bp XbaI/EcoRI-cut downstream DNA fragment were cloned into HindIII/EcoRI-cut pK19mobsacB [27] to generate pK-ΔoxyRcat.

(vi) Construction of Δcat allele for C. glutamicum ATCC13032. A 1036-bp upstream DNA fragment extending from nucleotide position −964 to +72 relative to the cat initiation codon was PCR amplified from C. glutamicum genomic DNA by PCR using the forward primer cg-cat-F1 (5′-GGTCCATTGTGTCAGGAA-3′, with the underlined mutations to introduce the PstI site shown in bold font) and cg-cat-R1 (5′-GGTTTCGAGAAAGCTTTACCTC-3′) containing the HindIII site shown in bold font). A 1026-bp downstream flanking region extending from −34 to +992 relative to the cat stop codon was PCR amplified using the forward primer cg-cat-F2 (5′-GGTCGAGAAAGCTTTACCTC-3′, with the underlined mutations to introduce the HindIII site shown in bold font) and cg-cat-R2 (5′-CCCTCAGCTTGTACGGAA-3′, with the underlined mutations to introduce the XbaI site shown in bold font). The 1036-bp PstI/HindIII-cut upstream DNA fragment and the 1026-bp HindIII/XbaI-cut downstream DNA fragment were cloned into PstI/XbaI-cut pK19mobsacB [27] to generate pK-ΔoxyRcat.

(vii) Construction of Δcat allele for C. glutamicum ATCC13032. A 1036-bp upstream DNA fragment extending from nucleotide position −964 to +72 relative to the cat initiation codon was PCR amplified from C. glutamicum genomic DNA by PCR using the forward primer cg-cat-F1 (5′-GGTCCATTGTGTCAGGAA-3′, with the underlined mutations to introduce the PstI site shown in bold font) and cg-cat-R1 (5′-GGTTTCGAGAAAGCTTTACCTC-3′) containing the HindIII site shown in bold font). A 1026-bp downstream flanking region extending from −34 to +992 relative to the cat stop codon was PCR amplified using the forward primer cg-cat-F2 (5′-GGTCGAGAAAGCTTTACCTC-3′, with the underlined mutations to introduce the HindIII site shown in bold font) and cg-cat-R2 (5′-CCCTCAGCTTGTACGGAA-3′, with the underlined mutations to introduce the XbaI site shown in bold font). The 1036-bp PstI/HindIII-cut upstream DNA fragment and the 1026-bp HindIII/XbaI-cut downstream DNA fragment were cloned into PstI/XbaI-cut pK19mobsacB [27] to generate pK-ΔoxyRcat.

(viii) The pK19mobsacB-derived plasmids described above were transformed into E. coli S17-1 [28] and mobilized by conjugation into C. diphtheriae C7(β), C. diphtheriae NCTC13129, or C. glutamicum ATCC13032, as appropriate. Kanamycin was used to select for co-integrates, and sucrose counter-selection was used to identify resolved co-integrates which were then screened to identify isolates carrying the desired ΔoxyR or Δcat alleles. Details for these procedures were described previously [29].

Agar diffusion growth inhibition assays for susceptibility to H2O2

Bacteria were cultured in low-iron PGT medium or PGT medium with 10 μM FeCl3, as indicated, and were harvested in exponential phase (A600 between 0.8 and 1.5). Samples containing approximately 107 bacteria were spread on the surface of HIA agar plates. A 10 μl drop of 1 M H2O2 (for C. diphtheriae strains) or 4 M H2O2 (for C. glutamicum strains) was spotted onto the center of the plate, and the diameter of the growth inhibition zone was measured after overnight incubation.

Construction and use of cat::lacZ reporter plasmids

Nov1/XbaI-cut pK-PIM [30] was treated with the Klenow fragment of DNA polymerase and ligated with the blunt-ended 3.6-kb Smal/XmnI DNA fragment from pSPZ, which contains the lacZ gene [31], to generate pKPL. DNA fragments containing 127-bp, 227-bp, or 327-bp from the regulatory region upstream from the cat gene were amplified from chromosomal DNA of C. diphtheriae C7(β) by PCR using the forward primer cat-F5 (5′-AAGCTTGGTAAATCCTGGAAGAAA-3′, added HindIII sequence in bold font), cat-F6 (5′-AAGCTTGGTAAATCCTGGAAGAAA-3′, added HindIII sequence in bold font), cat-F7 (5′-AAGCTTGGTAAATCCTGGAAGAAA-3′, added HindIII sequence in bold font), respectively, and the reverse primer cat-R5 (5′-AAGCTTGGTAAATCCTGGAAGAAA-3′, with the underlined mutations to introduce the XbaI site shown in bold font). The expected sequences of the PCR products were confirmed by DNA sequence analyses. Each HindIII/XbaI-cut PCR product was cloned, together with the SpeI/HindIII-cut 2.0 kb DNA (5′-Sp) [32], into SpeI/XbaI-cut pKPL to generate pPL-cat100, pPL-cat200, and pPL-cat500. Each of these plasmids contains transcription and translation stops in the upstream DNA fragment that block any fortuitous transcriptional or translational read-through from the vector DNA into cat. Plasmid pPL-Ω contains the Ω DNA upstream from the promoterless lacZ.
was normalized to the abundance of \( \text{dnaE} \) transcripts for each bacterial strain. Gene expression was measured with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Total RNA from \( C. diphtheriae \) was purified bacterial RNA, Cepheid Omnimix bead (Takara, Japan), DNaseI (Promega, Madison, WI). RNA concentration was measured by optical density at 260 nm and polyacrylamide sequencing gel, and the start site for the cat transcript was deduced from the experimentally determined sequence of the complementary primer extension product.

**Production and purification of recombinant  \( C. diphtheriae \) OxyR and \( E. coli \) OxyR**

For cloning into the C-terminal His6-fusion plasmid pET-22b (+) (Novagen, Madison, WI), the initiation and stop codons of the oxyR gene from \( C. diphtheriae \) C7/β were modified to introduce Nol and Xhol sites, respectively. The forward primer oxyR-F5 (5' ATAATAAGCCATGGGCAATA-3'), with the underlined mutation in the oxyR start codon to introduce the Nol site shown in bold font) was used in PCR with \( C. diphtheriae \) C7/β genomic DNA as template and the reverse primer oxyR-R4 (5'TTATGCTAATCCGAGCGCGAT-3', with the underlined mutations in and near the XhoI stop codon to introduce the XhoI site shown in bold font). The Nol/XhoI-cut PCR product was cloned into Nol/XhoI-cut pET-22b (+) to generate pET-OxyR. Sequence analysis confirmed that the inserted fragment was in the correct reading frame and that no unexpected mutations were present in the amplified DNA.

**RNA isolation and quantitative reverse-transcriptase PCR (qRT-PCR)**

RNA isolation was performed as described previously [21]. To study effects of \( H_2O_2 \) on abundance of cat transcripts, \( C. diphtheriae \) C7/β was grown in PGT medium with 10 \( \mu \)M FeCl₃ to mid exponential phase (\( A600 \) between 2.5 and 3.0) and treated with \( H_2O_2 \) at a final concentration of 1 \( \mu \)M, 100 \( \mu \)M, 1 \( \mu \)M or 10 \( \mu \)M for 5 min or 30 min. To compare the abundance of cat transcripts in wild-type C7/β and C7/β ΔoxyR at different stages of growth, each bacterial strain was grown in low-iron PGT medium or PGT medium with 10 \( \mu \)M FeCl₃ and harvested either during exponential phase (\( A600 \) between 0.8 and 1.5) or early stationary phase (\( A600 \) between 6 and 7). Cells were suspended in acid phosphatase and disrupted by shaking three times for 1 min for each treatment in a bead-beater. After centrifugation, RNA was extracted from the supernatant with acidified phenol/chloroform, precipitated with ethanol, and treated with RNase-free DNaseI (Promega, Madison, WI). RNA concentration was measured with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE).

Each cDNA synthesis reaction mixture contained 1 \( \mu \)g of purified bacterial RNA, Cepheid Omnimix bead (Takara, Japan), specific cat primers, and a cat probe with 5'-6-carboxyfluorescein and 3' black hole quencher 1 modifications. Reactions were performed in a Cepheid Smartcycler II (Cepheid, Sunnyvale, CA). Genomic DNA containing the gene of interest was used to generate a standard curve for each probe using identical experimental conditions. The abundance of the cat transcripts was normalized to the abundance \( \text{duaE} \) transcripts for each RNA sample.

**Assays for catalase activity**

Spectrophotometric assays for catalase activity were performed by monitoring the rate of degradation of \( H_2O_2 \) at A240 over 90 sec at room temperature. Each 1 ml reaction mixture contained 20 \( \mu \)g of crude soluble bacterial protein in 100 mM potassium phosphate, pH 7.4. The reactions were started by adding \( H_2O_2 \) to a final concentration of 10 mM. Catalase specific activity is expressed as units corresponding to 1 mmol of \( H_2O_2 \) decomposed/mg protein/min [34]. Staining of native polyacrylamide (12%) gels for catalase activity was performed as described previously [35]. Samples of crude bacterial extracts containing 20 \( \mu \)g of protein were electrophoretically separated on native polyacrylamide gels. Each gel was rinsed with distilled water and soaked for 10 min in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM \( H_2O_2 \). The gel was washed once again with distilled water, and then soaked for several minutes in 50 ml of a solution containing 1% ferric chloride and 1% potassium ferricyanide to allow the background color to develop. No color develops at the position(s) where catalase is present and \( H_2O_2 \) is degraded. Protein concentrations were determined by a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as the standard.

**OxyR Represses Cat Transcription in \( C. diphtheriae \)**

Primer extension analysis of cat transcripts

Primer extension analysis of cat transcripts was performed with reverse primer cat-PE1 (5’-GGTCAGGATGGAATCGACTG-3’), an anti-sense sequence that pairs approximately with codons 4–10 of the cat transcript. The reverse primer was 5'-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega, Madison, WI). Total RNA from \( C. diphtheriae \) C7/β was purified from samples harvested in the exponential phase of growth (\( A600 \) between 0.8 and 1.5) and used as template for dideoxy-termination sequencing with the Thermo Sequenase™ cycle sequencing kit (Amersham pharmacia biotech, Cleveland, OH). The product of the primer extension reaction was analyzed on an 8.3 M urea-8% polyacrylamide sequencing gel, and the start site for the cat transcript was deduced from the experimentally determined sequence of the complementary primer extension product.
medium, induced during exponential growth phase with IPTG, and the C-terminally His6-tagged E. coli OxyR protein was purified as described above for the comparable recombinant C. diphtheriae OxyR protein.

Gel-mobility shift assays

The 110-bp overlapping DNA probes designated (a), (b), and (c) are EcoRI fragments from the pT-cat A, pT-cat B, and pT-cat C plasmids described below. C. diphtheriae C7\(\beta\) genomic DNA was amplified by PCR with forward primer cat-F12 (5'-TCAT- GAACTCTTAGCACGTG-3') and reverse primer cat-R5 (5'- CCTATCAAATTGGAATTGCTG-3') to produce fragment (a), with forward primer cat-F13 (5'-CAAGCGGCGAGTGGCGC-3') and cat-R10 (5'-CAACTAAGGTTGAATTGCTG-3') to produce fragment (b), and with forward primer cat-F14 (5'-TAAGCTAT-TAATCGATTC-3') and reverse primer cat-R11 (5'-TGAG-GAGCTGCTTGAGTCCG-3') to produce fragment (c). Fragments (a), (b), and (c) were cloned into the pCR2.1 TOPO plasmids described below.

Sequenase\textsuperscript{TM} cycle sequencing kit (Amersham pharmacia biotech, Piscataway, NJ) was used to determine the corresponding regions of the genome sequence cloned fragments (a), (b), and (c) were determined and shown to be identical with the corresponding regions of the genome sequence from C. diphtheriae NCTC13129. The EcoRI fragments corresponding to probes (a), (b), and (c) were excised from pT-cat A, pT-cat B, and pT-cat C, purified, and labeled with [\(\gamma\)^32P]ATP using T4 polynucleotide kinase (Promega, Madison, WI). The DNA probes (approximately 10\(^6\) cpmp) were incubated at RT with varying amounts of purified OxyR protein from C. diphtheriae or E. coli in the binding reaction buffer reported previously [36]. When noted, 200 mM of diethiothreitol (DTT) was added to the reaction mixtures to reduce any disulfide bonds between cysteine residues in the OxyR protein. After incubation for 20-min, the reaction mixtures were analyzed using 5% non-denaturing polyacrylamide gels as described previously [36].

DINase footprinting assay

A 244-bp DNA fragment was amplified from C. diphtheriae C7\(\beta\) genomic DNA by PCR using the cat-F13 forward primer described previously and the cat-R12 reverse primer (5'-CTTGTTCAG- GATTTGATCGAC-3') which had been 5'-labeled with [\(\gamma\)^32P]ATP and T4 polynucleotide kinase (Promega, Madison, WI). The resulting 32P-labeled DNA probe (approximately 10\(^5\) cpmp, final concentration 0.12 nM) was incubated at RT with varying amounts of purified OxyR protein from C. diphtheriae or E. coli in the binding reaction buffer reported previously [36]. When noted, 200 mM of diethiothreitol (DTT) was added to the reaction mixtures to reduce any disulfide bonds between cysteine residues in the OxyR protein. After incubation for 20-min, each reaction mixture was digested with 0.5 U of DINAseI (Promega, Madison, WI) for 2 min at 37\(^\circ\)C, and the products were separated by electrophoresis on a 5% non-denaturing polyacrylamide sequencing gel [36]. The nucleotide sequence was determined in a parallel reaction by the dideoxy-termination method using as template the pT-cat A toplasmid which contains the cloned 244-bp fragment used for footprinting, the 5’-32P-labeled cat-R12 reverse primer described above, and the Thermo Sequenase\textsuperscript{\textregistered} cycle sequencing kit (Amersham pharmacia biotech, Cleveland, OH).

Western immunoblot assay

E. coli GC4468 or JL102 strains (harboring plasmids as indicated in the text) were grown in LB medium at 37\(^\circ\)C and harvested during the exponential phase of growth (A600 between 0.7 and 0.9). C. diphtheriae C7\(\beta\) or C7\(\beta\) OxyR strains (harboring plasmids as indicated in the text) were grown in PGT medium with 10 \(\mu\)M FeCl\(_3\) and harvested during the exponential phase of growth (A600 between 1.0 and 1.5). Cells were disrupted using an ultrasonic liquid process (Ecoli or a bead-beater (C. diphtheriae), and electrophoresis (SDS-PAGE, 12% polyacrylamide) and electro-blotting of proteins were performed as described previously [26]. Each blot was treated with a 1/2000 dilution of polyclonal rabbit antibody against E.coli OxyR (kindly provided by Dr. Giesela Storz) followed by a 1/10,000 dilution of goat anti-rabbit IgG (HRP), conjugated with horseradish peroxidase (HRP), and then visualized using a SuperSignal West Dura chemiluminescent substrate detection system supplied by Pierce. Protein concentrations of extracts were determined by NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE).

Results

The role of catalase in resistance to \(\text{H}_2\text{O}_2\)-induced oxidative stress in C. diphtheriae

C. diphtheriae is a catalase-positive gram-positive bacterium. As a baseline for studying the role of catalase in resistance of C. diphtheriae to \(\text{H}_2\text{O}_2\)-induced oxidative stress, we determined the effects of \(\text{H}_2\text{O}_2\) on growth (Fig. 1A) and viability (Fig. 1B) of wild type C. diphtheriae C7\(\beta\). For bacteria growing in PGT medium with 10 \(\mu\)M FeCl\(_3\), adding \(\text{H}_2\text{O}_2\) at a final concentration of 10 mM during the exponential phase of growth did not affect either growth rate or bacterial viability after 6 hours of exposure. Increasing the \(\text{H}_2\text{O}_2\) concentration to 100 mM caused a lag of approximately 2 hours before resumption of normal growth but had little effect on viability after 6 hours of exposure. In contrast, when \(\text{H}_2\text{O}_2\) was added at 300 mM, the wild-type cells showed complete growth arrest and no viable cells were present after 6 hours exposure. We observed similar effects of \(\text{H}_2\text{O}_2\) on growth and viability of wild type C. diphtheriae C7\(\beta\) when the experiments were repeated in low-iron PGT medium or in HIBTM medium (data not shown). These results show that wild type C. diphtheriae C7\(\beta\) exhibits substantial tolerance to \(\text{H}_2\text{O}_2\), presumably by degrading \(\text{H}_2\text{O}_2\) to non-toxic products.

The most obvious candidate enzymes for detoxifying \(\text{H}_2\text{O}_2\) are catalases and peroxidases. A search of the published C. diphtheriae NCTC13129 genome sequence for homologs of catalase identified a single gene annotated as cat (DIP0281) (http://www.ncbi.nlm.nih.gov/gene/2649075). The cat gene ORF is separated by a 127-bp intervening sequence from the divergently oriented sigC ORF, which encodes an RNA polymerase sigma factor presumed to be involved in responses to environmental stress (Fig. 2A). We constructed an in-frame deletion in the cat gene of C. diphtheriae C7\(\beta\) by allelic exchange, and we used this \(\Delta\text{cat}\) mutant strain to examine the role of catalase in the resistance C. diphtheriae to \(\text{H}_2\text{O}_2\). In contrast with the results described previously with wild type C7\(\beta\), addition of \(\text{H}_2\text{O}_2\) at 10 mM, 100 mM or 300 mM to exponential phase cultures of the C7\(\beta\) \(\Delta\text{cat}\) mutant resulted in immediate cessation of growth and complete loss of viability after 6 hours of exposure (Figs. 1A and 1B). When methyl viologen (MV, a superoxide radical generating agent) was spotted on agar plates inoculated with sufficient numbers of wild type C. diphtheriae C7\(\beta\) or the C7\(\beta\) \(\Delta\text{cat}\) mutant to produce confluent lawns during subsequent incubation, no significant differences were seen in the diameters of the zones of inhibition that developed (data not shown). These results indicate that catalase confers resistance to \(\text{H}_2\text{O}_2\)-induced stress, but not to MV-induced stress, in C. diphtheriae.
Effects of growth phase and H$_2$O$_2$ on activity of the cat promoter and catalase

As an initial method to localize the cat promoter and measure its activity, we constructed and characterized a set of cat::lacZ transcriptional fusion reporter plasmids (Fig. 2A). Plasmids pPL-cat100, pPL-cat200, and pPL-cat300 contain, respectively, the 100-bp segment of the sigC-cat intergenic sequence immediately upstream from the cat ORF and the longer 200-bp and 300-bp DNA sequences which extend further upstream from the cat ORF.

For C. diphtheriae C7(β) strains harboring either pPL-cat100, pPL-cat200, or pPL-cat300, the β-galactosidase reporter activity was significantly greater (approximately 1.8-fold) during the exponential phase of growth than during early stationary phase (P<.05) (Fig. 2B). The control plasmid pPLΩ, which does not contain any of the sigC-cat intergenic sequence, showed no β-galactosidase reporter activity. These data show that the functional cat promoter is located within the 100-bp segment of the sigC-cat intergenic sequence immediately upstream from the cat ORF and that the cat promoter is slightly but significantly more active during exponential growth than in stationary phase.

We measured catalase in exponential phase and early stationary phase cultures of wild type C. diphtheriae C7(β) by preparing extracts, subjecting them to electrophoresis on non-denaturing polyacrylamide gels, and performing in gel assays for catalase activity (Fig. 2C). The catalase from wild-type C7(β) migrated as a single band on the non-denaturing polyacrylamide gels and appeared, from the relative intensity of the reaction signals, to have 2–3 fold greater activity in the bacteria harvested during exponential phase compared to the bacteria harvested in the early stationary phase. Additional assays showed that catalase from C. diphtheriae does not have peroxidase activity and that catalase activity did not differ significantly in bacteria grown under high-
lysate from wild type C7. Samples were analyzed by native PAGE followed by in gel assays for catalase activity, which appears as the single bright band in each lane containing 100 mM H2O2 vs. a control culture without added H2O2 (Fig. 3A).

In the H2O2–treated cultures, activity of the cat promoter in C. diphtheriae C7(β) harboring pPL-cat200 in PGT medium with 10 mM FeCl3, and we compared β-galactosidase activity in individual cultures at various times after adding 10 mM or 100 mM H2O2 vs. a control culture without added H2O2 (Fig. 3A). In the H2O2–treated cultures, activity of the β-galactosidase reporter for the cat promoter showed a trend toward slight and progressive decrease over time, but none of the differences between the treated cultures and the untreated control cultures was statistically significant (P>0.05). To confirm and extend these findings, we used qRT-PCR to measure directly the relative abundance of cat transcripts in untreated control cultures and in cultures exposed for 5 or 30 min to H2O2 at concentrations of 1 μM, 100 μM, 1 mM and 10 mM (Fig. 3B). After 5 minutes, the relative abundance of cat transcripts decreased slightly in all of the H2O2–treated cultures, but the differences in cat transcript abundance between the treated and untreated cultures were statistically significant only for the 100 μM and 1 mM H2O2 treatment groups (p<0.05). After 30 min, the relative abundance of cat transcripts in treated cultures at each concentration of H2O2 did not differ significantly from the untreated controls (p>0.5).

In gel assays for catalase activity were performed on extracts prepared from control cultures of wild-type C. diphtheriae C7(β) during the exponential growth phase and from replicate cultures exposed for 30 minutes to H2O2 at 1 μM, 100 μM, 1 mM or 10 mM (Fig. 3C). By image analysis, catalase activity in the cultures treated with 1 μM, 100 μM, 1 mM and 10 mM H2O2 was estimated to be 87%, 85%, 92% and 96% of the catalase activity in the untreated control cultures, respectively. Extending the duration of H2O2 exposure to 2 h also failed to induce catalase activity (data not shown). In summary, treating C. diphtheriae C7(β) with H2O2 at concentrations from 1 μM to 10 mM resulted in insignificant to small but significant decreases in cat promoter activity and slight decreases in catalase activity. These findings differed dramatically from published results with E. coli showing that exposure to H2O2 at concentrations ranging from 100 μM to 1 mM activates transcription of katG and production of catalase [38]. Our results provide no evidence that cat transcription or catalase production in C. diphtheriae is activated by exposure to H2O2.

The role of OxyR in regulating the cat promoter and catalase in C. diphtheriae

Examination of the published genome sequence for C. diphtheriae NCTC13129 [39] identified one ORF (DIP1421) that encodes a putative transcriptional regulator similar to the H2O2-inducible OxyR from Erwinia carotovora. The predicted amino acid sequence of the C. diphtheriae OxyR encoded by DIP1421 is 100% identical.
Figure 3. Exposure of wild-type *C. diphtheriae* C7(β) to H₂O₂ had little effect on cat promoter activity or catalase activity. A: Replicate cultures of *C. diphtheriae* C7(β) harboring pPL-cat200 were grown in high-iron PGT medium to exponential phase, and β-galactosidase activities were measured subsequently at the indicated times in a control without H₂O₂ (top histogram, open bars), and after addition of 10 mM H₂O₂ (middle histogram, shaded bars) or treatment for 30 min with 10 mM or 100 mM H₂O₂ as it did without exposure to H₂O₂, and (Fig. 1A), the C7(β) or 100 mM H₂O₂ (bottom histogram, hatched bars). The error bars indicate standard deviations. B: Relative abundance of cat transcripts was determined with RNA extracted from mid-exponential phase cultures of wild type *C. diphtheriae* C7(β) after no treatment (open bars) or treatment for 5 min or 30 min with 1 mM H₂O₂ (shaded bars), 100 µM H₂O₂ (hatched bars), 1 mM H₂O₂ (dotted bars) or 10 mM H₂O₂ (lined bars). C: In gel assays for catalase were performed with extracts from mid-exponential phase cultures of wild type *C. diphtheriae* C7(β) after no treatment (lane 1) or treatment for 30 min with 1 mM H₂O₂ (lane 2), 100 µM H₂O₂ (lane 3), 1 mM H₂O₂ (lane 4) or 10 mM H₂O₂ (lane 5). Standard deviations were calculated from results of assays performed in triplicate.

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with the putative *C. glutamicum* OxyR, 40% identical and 62% similar with *Streptomyces coelicolor* OxyR, 33% identical and 62% similar with *E.coli* OxyR, and 27% identical and 47% similar with *N. gonorrhoeae* OxyR (Fig. S1A). Each of these proteins has a highly conserved DNA binding domain characteristic of the LysR family of regulatory proteins as well as two conserved cysteine residues (Cys206 and Cys215 in *C. diphtheriae* OxyR) homologous to the Cys199 and Cys208 residues in *E.coli* OxyR that participate in redox sensing. No gene encoding a homolog of the *E. coli* OxyS protein is present in the *C. diphtheriae* genome. The oxyR gene in *C. diphtheriae* is flanked on the upstream side by the divergently transcribed *dixA* gene (which encodes an iron-repressible polypeptide) and on the downstream side by the convergently transcribed DIP1422 gene (which encodes a putative membrane protein) (Fig. S1B).

We constructed an in-frame deletion mutation in the oxyR gene of *C. diphtheriae* C7(β) by allelic exchange (Fig. S1B). We examined susceptibility of the C7(β) ΔoxyR mutant to oxidative stress by comparing the effects on growth of H₂O₂ at final concentrations of 10 mM, 100 mM, and 300 mM in replicate exponential phase cultures vs. control cultures without exposure to H₂O₂. In striking contrast with the findings for wild type C7(β) shown previously (Fig. 1A), the C7(β) ΔoxyR mutant grew as well after exposure to 10 mM or 100 mM H₂O₂ as it did without exposure to H₂O₂, and it showed only a slight decrease in growth after exposure to 300 mM H₂O₂ (Fig. 4A). The tolerance of the C7(β) ΔoxyR mutant to H₂O₂ was comparable in low-iron PGT medium, PGT medium with 10 µM FeCl₃, and HIBTW medium (data not shown). Introducing a cloned wild type oxyR allele into the C7(β) ΔoxyR mutant by integrating the pK-PIM-oxyR(β) plasmid into a chromosomal attB site [30] complemented the ΔoxyR mutation and restored the growth phenotype after exposure to H₂O₂ to wild type (data not shown).

We used two different methods to demonstrate the effects of OxyR on activity of the cat promoter. First, we introduced the pPL-cat100, pPL-cat200, or pPL-cat300 reporter plasmid (or the pPLΔ control plasmid) into wild type *C. diphtheriae* C7(β) and the C7(β) ΔoxyR mutant and measured β-galactosidase activity as a surrogate for cat promoter activity during exponential growth of each strain in PGT medium with 10 µM FeCl₃ (Fig. 4B). Each of the C7(β) ΔoxyR derivatives exhibited a statistically significant 6–8 fold increase in β-galactosidase activity compared with the corresponding isogenic wild-type C7(β) derivative (P<0.05), and no β-galactosidase activity was present in C7(β) or C7(β) ΔoxyR harboring pPLΔ. To confirm and extend these findings, we used real-time quantitative RT-PCR assays to compare the relative abundance of cat transcripts in wild type C7(β) and the isogenic C7(β) ΔoxyR mutant during exponential growth (Fig. 4C) and early...
stationary phase (Fig. 4D), both in low-iron PGT medium and in PGT medium with 10 μM FeCl₃. The normalized abundance of cat transcripts was significantly greater by 6–8 fold in C7(#)DoxyR than in C7(#) under each set of growth conditions (P<0.05). For each strain, the normalized abundance of cat transcripts declined by two-fold or more during transition from the exponential growth phase to early stationary phase, confirming our previous finding using reporter plasmids in wild type C7(#) (Fig. 2B). Furthermore, in C7(#) and C7(#)DoxyR during exponential and early stationary phase growth, the normalized abundance of cat transcripts exhibited a small (two-fold or less) but significant (P<0.05) increase under high-iron vs. low-iron conditions.

We performed direct biochemical assays for catalase activity with extracts prepared from exponential phase cultures of C. diphtheriae C7(#), C7(#) Δkat, and C7(#) ΔoxyR grown in low-iron PGT medium and in PGT medium with 10 μM FeCl₃ (Fig. 5A). No catalase activity was detected in C7(#)Dcat, confirming that cat is the sole gene responsible for production of catalase in C. diphtheriae C7(#). The specific activity of catalase was approximately 15- to 20-fold greater in C7(#)DoxyR than in wild type C7(#), but growth in low-iron vs. high-iron medium had no significant effect on catalase activity (P>0.05). Analysis of these extracts by non-denaturing PAGE and in gel assays for catalase activity supported these conclusions (Fig. 5B). Taken together, these findings demonstrate that OxyR functions as a repressor of cat transcription, support the conclusion that regulation of catalase occurs primarily at the level of transcription in C. diphtheriae, and provide no evidence that OxyR functions as an activator of cat transcription in C. diphtheriae.

Both in wild type C7(#) and in C7(#)DoxyR, we found small but statistically significant (P<0.05) increases in the normalized abundance of cat transcripts under high iron vs. low-iron growth conditions (Figs. 4C and 4D) that were not accompanied by increases in catalase activity (Figs. 5A and 5B). These effects were independent of OxyR, since they occurred both in C7(#) and in C7(#)DoxyR.

We also showed by real-time quantitative RT-PCR assays that the relative abundance of sigC transcripts was about 1.6-fold greater in C7(#)DoxyR than in C7(#) during exponential phase growth in low-iron PGT medium, and about 2-fold greater in C7(#)DoxyR than in C7(#) during exponential phase growth in high-iron PGT medium (data not shown). These modest differences suggest that OxyR has little role in regulating transcription of sigC, notwithstanding the fact that cat and sigC
are separated only by a short intergenic region and are divergently transcribed (Fig. 2A).

Molecular analysis of OxyR interaction with the promoter in C. diphtheriae

Our previous experiments using pPL-cat100, pPL-cat200, and pPL-cat500 as reporters for cat promoter activity (Figs. 2B and Fig. 4B) showed that the cat promoter is located within the 100 bp segment of the sigC-cat intergenic region that lies immediately upstream from the cat ORF start codon. In an effort to predict a possible OxyR binding target by bio-informatic methods, we used the MEME program (http://meme.sdsc.edu/meme/) to search the sigC-cat intergenic region for homologs of the known 50 bp E.coli OxyR binding target (5’-CAATAAAGCAATGGTTAATTTGTAAGAATTACCTA-3’). The best match, extending from 52–103 bp upstream from the cat ORF start codon, was 75% similar to the E.coli OxyR binding target. As a first step toward testing whether C. diphtheriae OxyR can bind to this predicted site, we designed three overlapping 110 bp DNA probes for use in gel mobility shift assays. Probe (a), extending from nucleotide −113 to −22 with respect to the +1 nucleotide of the cat ORF start codon, was centered on the predicted binding site described above (Fig. 6A). Probe (b), extending from nucleotide −211 to −102, and probe (c), extending from nucleotide −51 to +59, lie immediately upstream and immediately downstream, respectively, from the predicted binding site described above (Fig. 6A). Gel mobility shift assays showed that purified C. diphtheriae OxyR bound to and shifted probe (a) but not probe (b) or probe (c) (Fig. 6B). The position of the shifted probe (a) did not change as the concentration of C. diphtheriae OxyR (calculated as monomer) added to the gel shift reaction mixture increased from 56 pM to 280 pM, suggesting that OxyR formed complexes with DNA of uniform stoichiometry within this range of experimental conditions. Purified C. diphtheriae OxyR shifted probe (a) to the same extent and to the same position whether or not 200 mM DTT was present in the gel shift reaction mixture. These experiments were performed on three different occasions, with the same results. These findings suggest that purified C. diphtheriae OxyR can bind to the cat promoter region either in its reduced or oxidized form.

Next, we mapped the 5’ end of the cat transcripts in total RNA isolated from exponential phase cultures of wild-type C. diphtheriae C7[β] and the C7[β] ΔoxyR mutant grown in low-iron PGT medium and in PGT medium with 10 μM FeCl3 (Fig. 7A). In both bacterial strains and in both growth media, the 5’ end of the cat transcript was the C residue at position −39 relative to the +1 position in the cat ORF start codon. The identity of the band corresponding to the primer extension product was greater with RNA from C7[β] ΔoxyR than from wild type C7[β], reflecting the greater abundance of cat transcripts in C7[β] ΔoxyR (Fig. 4C).

To define more precisely the binding target for C. diphtheriae OxyR in the cat promoter region, we performed DNaseI footprinting assays. Either in the presence or absence of 200 mM DTT, binding of OxyR protected a 46-bp AT-rich sequence extending from position −55 to −10 with respect to the +1 position in the cat ORF start codon (Fig. 7B). The extent of protection increased progressively as the concentration of OxyR in the reaction mixture increased from 0.28 nM to 2.8 nM. The OxyR binding site demonstrated by the DNaseI protection assays was immediately downstream from, but did not overlap with, the putative binding site predicted by the bio-informatics analysis described above based on homology with the binding site for E. coli OxyR.

The 46 bp sequence corresponding to the DNaseI footprint is shown in bold font in Fig. 7C. Both the G residue at position −22 that corresponds to the 3’ end of probe (a) and the T residue at position −51 that corresponds to the 5’ end of probe (c) lie within this DNaseI footprint. The upstream and downstream vertical lines inserted into the sequence in Fig. 7C show the location of the 5’ end of probe (c) and the 3’ end of probe (a), respectively. The ability of C. diphtheriae OxyR to shift the mobility of probe (a) (Fig. 6B) indicates that the 13 nucleotides at the 3’ end of the DNaseI footprint sequence are not required for OxyR binding to the cat promoter region, although they might contribute to optimal binding. Conversely, the failure of C. diphtheriae OxyR to shift the mobility of probe (c) (Fig. 6B) suggests that some or all of the 4 nucleotides at the 5’ end of the DNaseI footprint sequence might be required for OxyR binding. To investigate this possibility further, we used PCR to prepare a nested set of seven DNA amplicons that shared the downstream end of probe (c) at position +59 with respect to the relative to the +1 position in the cat ORF start codon but differed at their upstream ends. The shortest amplicon was identical to probe (c), and the six longer amplicons extended 2 bp, 4 bp, 9 bp, 14 bp, 19 bp or 29 bp further upstream to terminate at positions −53, −55, −60, −65, −70 or −80, respectively. In gel mobility shift assays, C. diphtheriae
OxyR failed to shift the mobility of the amplicon that was identical to probe (c) but did shift the mobility of all of the longer amplicons (data not shown). We conclude, therefore, that the sequence extending downstream from the T residue at position −53 in Fig. 7C contains the minimal essential binding site for C. diphtheriae OxyR.

Comparison of Δcat and ΔoxyR mutations in C. diphtheriae and C. glutamicum

C. diphtheriae C7[β] has been maintained in laboratories since the 1950s [24] and has been used by many investigators as a reference strain for research. To determine whether our results with C7[β] are representative of other isolates of C. diphtheriae or of another Corynebacterium species, we performed confirmatory studies with the recent clinical isolate C. diphtheriae NCTC13129 (used for the first genome sequence of this species) and with C. glutamicum ATCC13120 [a nonpathogenic Corynebacterium widely used in biotechnology]. For each of these reference strains, we constructed an isogenic Δcat and an isogenic ΔoxyR in-frame single deletion mutant, and we complemented the ΔoxyR mutant by integration of pK-PIM-oxyR<sub>C7[b]</sub> into a chromosomal attB site. We then compared the newly constructed wild-type, mutant and complementation variants of C. diphtheriae NCTC13129 and C. glutamicum ATCC13120 with the homologous C7[β] variants described previously. For this purpose, we used agar-diffusion growth-inhibition assays to test the tolerance of each of these bacterial variants to H₂O₂. Fig. 8A shows photographs of representative plates from tests with wild-type C7[β], with the isogenic Δcat and ΔoxyR mutants, and with the ΔoxyR mutant complemented with pK-PIM-oxyR<sub>C7[b]</sub>. Fig. 8B presents the results from quantitative measurements of the diameters of the growth inhibition zones from tests performed with the wild-type C. diphtheriae C7[β], C. diphtheriae NCTC13129 and C. glutamicum ATCC13120 strains and the mutant and complementation variants derived from them. In all cases, the Δcat mutants exhibited increased susceptibility to H₂O₂, the ΔoxyR mutants showed dramatically decreased susceptibility to H₂O₂, and the susceptibility to H₂O₂ of the complemented ΔoxyR mutants was comparable to that of their wild-type parental strains. Complementation tests with pK-PIM-oxyR plasmids carrying the wild-type oxyR allele from C. diphtheriae NCTC13129 or C. glutamicum ATCC13120 gave equivalent results to those described above with the pK-PIM-oxyR<sub>C7[b]</sub> plasmid (data not shown). These findings support the conclusions that, in both C. diphtheriae and C. glutamicum, catalase protects against H₂O₂–induced oxidative stress, oxyR functions as a repressor of cat, and oxyR clones from C. diphtheriae and C. glutamicum are expressed in a comparable manner.

C. diphtheriae OxyR and E.coli OxyR are not functionally equivalent

We used agar-diffusion growth-inhibition assays and complementation tests similar to those described above to evaluate the ability of the E. coli oxyR gene to substitute functionally for the C. diphtheriae oxyR gene. For tests in E. coli we used the wild-type strain GC4468 and its isogenic oxyR mutant strain JL102 [41].
OxyR Represses Cat Transcription in C. diphtheriae

Figure 7. Characterization of the oxyR promoter-operator region. A: The 5' end of the cat transcript was mapped by primer extension assays in wild-type C. diphtheriae C7(b) (lanes 1 and 2) and C7(b) ΔoxyR (lanes 3 and 4) grown to exponential phase in low-iron PGT medium (lanes 1 and 3) and high-iron PGT medium (lanes 2 and 4). The same 32P-labeled reverse primer was used for the sequence ladder (labels C, T, A, and G correspond to nucleotides in the sense strand of the cat gene) and the primer extension products (lanes 1, 2, 3 and 4). B: DNaseI footprint of the OxyR binding site in the cat regulatory region. Controls in lanes 1 and 6 contained no OxyR. Triangles at the top show increasing concentrations of OxyR (calculated as the monomer), which were 0.28 nM for lanes 2 and 7, 0.56 nM for lanes 3 and 8, 1.4 nM for lanes 4 and 9, and 2.8 nM for lanes 5 and 10. Lanes 1–5 had no DTT (labeled −DTT), and lanes 6–10 had 200 mM DTT (labeled +DTT). The vertical line shows the sequence that was protected by OxyR from DNaseI cleavage. The sequence ladder is labeled as in (A), and the arrow marks the C residue corresponding to the 5' end of the cat transcript. C: The DNA sequence includes the cat regulatory region and part of the cat ORF. The C residue at the 5' end of the cat transcript is marked by an up arrow; the putative ribosome-binding site (labeled S–D) is underlined; the putative −10 promoter hexamer (labeled −10) is italicized, bold and underlined; the putative −35 promoter hexamer (labeled −35) is italicized and underlined; and the 46-bp OxyR footprint (−55 to −10 relative to the start of the cat ORF) is bold. The upstream and downstream vertical lines within the OxyR footprint show the locations of the 5' end of probe (c) and the 3' end of probe (a), respectively. Three possible T-N11-A target motifs for OxyR binding within the OxyR footprint sequence are marked by filled diamonds, filled circles, and asterisks, respectively, above their 5' of probe (a), respectively. Three possible T-N11-A target motifs for OxyR binding within the OxyR footprint sequence are marked by filled diamonds, filled circles, and asterisks, respectively, above their 5' of probe (c) and the 3' end of probe (a), respectively. Three possible T-N11-A target motifs for OxyR binding within the OxyR footprint sequence are marked by filled diamonds, filled circles, and asterisks, respectively, above their 5' of probe (c).
with extracts from C7(β) or C7(β) ΔoxyR. E. coli OxyR was detected in extracts from C. diphtheriae C7(β) ΔoxyR harboring pK-PIM-oxyR Eco or pK-PIM-oxyR Eco2, and the amount of E. coli OxyR made in C7(β) ΔoxyR harboring pK-PIM-oxyR Eco2 was significantly greater that the amount detected in E. coli JL102 harboring pRK-oxyR Eco or pRK-oxyR Eco2. Taken together, these results demonstrated that E. coli OxyR encoded by a functional native or hybrid oxyR locus was produced in C. diphtheriae but failed to complement the ΔoxyR phenotype or substitute for C. diphtheriae OxyR as a repressor of transcription of the cat gene.

Finally, we used gel mobility shift assays to test directly the ability of purified OxyR from C. diphtheriae and purified OxyR from E. coli to bind to target sequences in DNA probe (a) (Fig. 6A) from the cat promoter region of C. diphtheriae (Fig. 10A) and to a 300 bp DNA fragment from the katG regulatory region of E. coli (Fig. 10B). The purified OxyR from C. diphtheriae bound to and shifted the mobility of probe (a), in agreement with results shown previously in Fig. 6B, but OxyR from E. coli failed to shift probe (a) either with or without 200 mM DTT in the reaction mix. Conversely, purified OxyR from E. coli bound the 300 bp E. coli target sequence and shifted its mobility progressively as the OxyR concentration increased, but no shift was seen with purified OxyR from C. diphtheriae either with or without 200 mM DTT in the reaction mix. These findings show that the purified OxyR proteins from C. diphtheriae and E. coli exhibit significant differences in their DNA binding specificities.

**Discussion**

There are few previous reports of enzymes and regulators involved in responses to oxidative stress in the human pathogen C. diphtheriae or the closely related soil bacterium C. glutamicum. The sodI gene that encodes the manganese-containing superoxide dismutase, an enzyme that scavenges superoxide radicals and protects against superoxide radical-induced oxidative damage, was cloned from C. glutamicum and characterized [23]. A previous study from our laboratory showed that insertional inactivation of the diphtheria toxin repressor gene (dtxR) in C. diphtheriae results in increased susceptibility to growth inhibition and killing following...
exposure to H$_2$O$_2$ [31]. In addition, exposure of *C. diphtheriae* to several stressors, including acid, cold, heat, ethanol or SDS, but not exposure to H$_2$O$_2$, increased transcription of the contiguous *sigB* and *dtxR* genes from the promoter located just upstream from *sigB* [21]. These studies provided initial evidence for metabolic interactions between *dtxR*, the iron-regulon and several non-related stress responses in *C. diphtheriae*.

In the current study, we chose two *C. diphtheriae* reference strains and one *C. glutamicum* reference strain for studies on the role of catalase in resistance to H$_2$O$_2$ and the role of OxyR, a member of the LysR family of bacterial regulatory proteins, in regulation of catalase (*cat*) gene expression. Published genome sequences for *C. diphtheriae* and *C. glutamicum* have a single gene for catalase [23,43] and we confirmed production of a single catalase enzyme in *C. diphtheriae* by biochemical analysis (Figs. 2C and 5B.). We showed both by use of *cat::lacZ* reporter plasmids (Figs. 2B and 4B) and by direct quantitative RT-PCR assays of *cat* transcript abundance that OxyR strongly represses *cat* transcription in *C. diphtheriae* by an H$_2$O$_2$-independent mechanism (Figs. 4C and 4D). Increased production of catalase by *D*$_{oxyR}$ mutants of *C. diphtheriae* and *C. glutamicum* conferred a dramatically increased level of resistance to H$_2$O$_2$ (Figs. 8A and 8B). Transcription of *cat* in *C. diphtheriae* decreased by about 2-fold during transition from the exponential growth phase to stationary phase (Figs. 2B, 4C and 4D).

Figure 9. OxyR from *E. coli* does not complement the ΔoxyR mutation in *C. diphtheriae*. A: The phenotypes of wild type *E. coli* strain GC4468 and its isogenic ΔoxyR derivative JL102 in H$_2$O$_2$ agar-diffusion growth-inhibition assays are shown. The mutant phenotype of *E. coli* JL102 was complemented by the cloned *E. coli* oxyR gene expressed either from its native *E. coli* oxyR promoter (in pRK-oxyR Eco) or from the heterologous *C. diphtheriae* oxyR promoter (in pRK-oxyR Eco2). B: The phenotypes of wild type *C. diphtheriae* C7(β) containing pK-PIM and its isogenic ΔoxyR derivative containing the pK-PIM are shown. *C. diphtheriae* OxyR expressed from its native promoter in the pK-PIM-oxyR$_{C7}$ clone complemented the mutant phenotype of C7(β) ΔoxyR. In contrast, *E. coli* OxyR expressed either from its native promoter in pK-PIM-oxyR Eco or from the heterologous *C. diphtheriae* oxyR promoter in pK-PIM-oxyR Eco2 failed to complement the mutant phenotype of C7(β) ΔoxyR. C: SDS-PAGE and Western blot analysis with rabbit antiserum against *E.coli* OxyR was performed on extracts from exponential phase cultures of the following bacterial stains: *E.coli* GC4468 (lane 1), *E.coli* JL102 (lane 2), *E.coli* JL102 harboring pRK-oxyR Eco (lane 3), *E.coli* JL102 harboring pRK-oxyR Eco2 (lane 4), *C. diphtheriae* C7(β) containing pK-PIM (lane 5), *C. diphtheriae* C7(β) ΔoxyR containing pK-PIM (lane 6), *C. diphtheriae* C7(β) ΔoxyR containing pK-PIM-oxyR$_{C7}$ (lane 7), *C. diphtheriae* C7(β) ΔoxyR containing pK-PIM-oxyR Eco (lane 8), and *C. diphtheriae* C7(β) ΔoxyR containing pK-PIM-oxyR Eco2 (lane 9). Precision plus protein dual color standard (Bio-Rad) was used for molecular mass standards.

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Transcription of cat in C. diphtheriae was slightly greater under high-iron growth conditions than under low-iron growth conditions, but the difference was statistically significant (Figs. 4C and 4D). We believe it is unlikely that DtxR directly mediates this effect of iron on transcription of cat, because the sigC-cat intergenic region contains no sequences that closely match the 19 bp consensus sequence for DtxR-binding. Molecular characterization of this effect of iron on cat transcription will require additional experiments that are beyond the scope of the current study. We did not identify other environmental signals that regulated cat transcription. We found no evidence that exposure of C. diphtheriae to H₂O₂ stimulates transcription of cat. In contrast, transcription of the catalase gene (katG) in E. coli during exponential growth is activated by OxyR in response to H₂O₂, and katG transcription during stationary phase is controlled by the starvation-induced sigma factor RpoS [44]. This mechanism of regulating cat transcription during stationary phase appears unlikely in C. diphtheriae or C. glutamicum, because their genomes do not encode rpoS [23,43].

Primer extension analysis showed that a (C) residue located 39 nucleotides upstream from the cat ORF is the transcriptional start (Fig. 7A). A recent study characterized the sigma factors of C. glutamicum and features of the specific promoters that they recognize [45]. This analysis of 159 sequences of presumed σ²⁷-dependent promoters in C. glutamicum revealed a consensus sequence of TANAAT for the −10 hexamer, GNTANANTA for the extended −10 region, and TTG/A/C/C/A for the −35 hexamer in the subset of the corynebacterial promoters for which a −35 sequence was identified. Inspection of the sequence upstream from the cat transcriptional start site (Fig. 7C) revealed a putative −10 promoter region (TTAACGT), which contains all three of the highly conserved T, A and T residues in the first, second and sixth positions of the −10 consensus sequence). Furthermore, this putative −10 hexamer is properly located with respect to the −35 promoter region. In addition, a consensus Shine-Dalgarno ribosome binding site (AGGAGG, labeled S–D in Fig. 7C) is present at the proper location upstream from the cat ORF. Although future studies will be needed to confirm the functional importance of each of these features for cat promoter activity, their presence provides a high degree of confidence that this region does represent the cat promoter.

DNaseI footprinting assays identified a 46 bp region protected by OxyR binding that extends from −55 to −10 nucleotides with respect to the +1 nucleotide in the cat ORF. This putative OxyR binding site overlaps with the putative −10 hexamer of the cat promoter, the start site for the cat transcript, and part of the putative ribosome binding site. Binding to this region is fully consistent with repression of cat transcription by OxyR, and a previous study in Pseudomonas putida showed that the LysR-type regulator CatR represses transcription of the catR gene by binding to a region located downstream from the start site of the catR transcript and overlaps with the ribosome binding site [46].

The OxyR proteins from C. diphtheriae and C. glutamicum are members of the LysR family of transcriptional regulators. A characteristic feature of binding sites for LysR-type bacterial regulatory proteins is the presence of one or more highly conserved T-N11-A motifs, each of which may serve as the core for an inverted repeat structure of variable complexity [47]. Examination of the sequence of the OxyR binding site identified by DNaseI footprinting of the cat promoter region of C. diphtheriae
reveals three putative T-N11-A motifs (Fig. 7C). The upstream putative T-N11-A motif (marked with filled diamonds above the conserved T and A residues at positions −53 and −41 with respect to the +1 nucleotide in the cat ORF) completely overlaps the putative −10 hexamer of the cat. The two partially overlapping downstream putative T-N11-A motifs (marked with filled circles above the conserved T and A residues at positions −36 and −24 and with bold asterisks above the conserved T and A residues at positions −25 and −13) are located between the cat transcript start site and the putative ribosome binding site. The horizontal dashed arrows in Fig. 7C mark short palindromic sequences that are associated with, but not always precisely centered on, these putative T-N11-A motifs. Future studies will be required to test directly the functional roles of each of these putative T-N11-A motifs and their associated palindromic sequences in repression of cat transcription by C. diphtheriae OxyR.

We used complementation tests and gel mobility shift assays to compare the function of the OxyR regulator from C. diphtheriae with the well-characterized OxyR regulator from E. coli, which was previously shown to act both as a sensor of H$_2$O$_2$-induced oxidative stress and as transcriptional activator of the catalase gene (katG) in E. coli [10]. The cloned oxyR gene from C. diphtheriae (which encodes a protein identical to C. glutamicum OxyR) fully complemented the ΔoxyR mutation in C. diphtheriae or C. glutamicum (Figs. 8A and 8B). In contrast, the cloned E. coli oxyR gene was shown to be functional in E. coli and to direct the production of the identical E. coli OxyR protein in C. diphtheriae (Fig. 9). These findings indicate that the OxyR proteins from C. diphtheriae and E. coli have distinct functional specificities, and differences in their ability to interact with OxyR binding sequences from C. diphtheriae and E. coli were confirmed by gel mobility shift assays (Figs. 10A and 10B).

Bacterial regulatory proteins in the LysR family typically form oligomers. The biologically active form of LysR regulators, including the prototypical OxyR from E. coli, is most often tetrameric, although active octamers or dimers have been described in some cases [48,49]. Fig. S1 shows that primary structure and domain organization of OxyR from C. diphtheriae and C. glutamicum are homologous with those of other, better characterized, OxyR proteins from a diverse group of bacterial species. We performed limited experiments to examine the oligomeric state of the purified recombinant His6-tagged that we prepared in this study. On a gel filtration chromatography column standardized with several other reference proteins, our C. diphtheriae OxyR migrated as a single peak with a mobility consistent with the predicted mass of a trimer (data not shown), but we could not rule out an anomalous mobility because of weak interactions with the gel matrix or other unknown factors. We also made limited but unsuccessful attempts to determine the oligomeric state of C. diphtheriae OxyR by several protein cross-linking methods (data not shown). Therefore, for this study, we expressed the concentration of C. diphtheriae OxyR used in individual experiments as monomer equivalents, and additional future studies will be needed establish definitively the oligomeric state(s) of biologically active C. diphtheriae OxyR.

In summary, our results demonstrate that catalase is the effector for defense against H$_2$O$_2$-induced oxidative stress in C. diphtheriae and C. glutamicum. They demonstrate that OxyR functions in an H$_2$O$_2$-independent manner as a repressor of cat transcription in C. diphtheriae, and they provide initial insights into the organization of the cat promoter and the molecular mechanisms for its interaction with OxyR in C. diphtheriae. Many questions about the role of OxyR in corynebacteria remain to be examined. For example, does corynebacterial OxyR function as a global transcriptional regulator, and if so what promoters (other than the cat promoter) does it repress or activate in corynebacteria? Does corynebacterial OxyR function as a sensor of oxidative stress in corynebacteria, and if so what molecular mechanisms determine the preferential activity of, oxidized or reduced forms of corynebacterial OxyR. Does the regulatory activity of corynebacterial OxyR at some promoters involve interactions with small molecule co-regulators, and if so what is the nature and functional role of such co-regulators. The initial characterization of corynebacterial OxyR reported here should serve as a sound basis for investigating such questions in future studies.

Supporting Information

Figure S1 OxyR sequence and organization of the oxyR locus in C. diphtheriae. A: Alignment of predicted amino acid sequences of OxyR from Corynebacterium diphtheriae, Corynebacterium glutamicum, Streptomyces coelicolor, Escherichia coli, and Neisseria gonorrhoeae, assembled using the Clustal W program. The predicted helix-turn-helix motif (HTH) near the amino terminus is indicated by the dotted line; the predicted DNA binding domain lies between the two bent arrows; and the conserved cysteine residues represented by C206 and C215 in OxyR from C. diphtheriae or C. glutamicum are indicated by asterisks. The numbering of amino acids is shown on the right side. B: Organization of the oxyR locus in wild type C. diphtheriae C7[β] and in the isogenic C7[β] ΔoxyR mutant. The grey arrow shows the orientation of the oxyR ORF, and the white arrows show the orientations of the flanking dnaA ORF (which encodes an iron-repressible polypeptide) and the DIP1422 ORF (which encodes a putative membrane protein). The ORF for the ΔoxyR allele, which contains an in-frame deletion, is shorter and is shown to scale in the lower diagram. (TIF)

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Author Contributions

Conceived and designed the experiments: JK RKH. Performed the experiments: JK. Analyzed the data: JK RKH. Contributed reagents/materials/analysis tools: JK RKH. Wrote the paper: JK RKH.

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