The RclR Protein Is a Reactive Chlorine-specific Transcription Factor in Escherichia coli*

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Background: Reactive chlorine compounds are important natural antimicrobials produced by the immune system.

Results: Reactive chlorine treatment leads to RclR cysteine oxidation, activation of DNA binding, and expression of RclR-controlled genes.

Conclusion: RclR is a transcriptional activator that responds specifically to reactive chlorine.

Significance: Understanding reactive chlorine responses is important for understanding interactions between bacteria and the host immune system.

Reactive chlorine species (RCS) such as hypochlorous acid are powerful antimicrobial oxidants. Used extensively for disinfection in household and industrial settings (i.e., as bleach), RCS are also naturally generated in high quantities during the innate immune response. Bacterial responses to RCS are complex and differ substantially from the well characterized responses to other physiologically relevant oxidants, like peroxide or superoxide. Several RCS-sensitive transcription factors have been identified in bacteria, but most of them respond to multiple stressors whose damaging effects overlap with those of RCS, including reactive oxygen species and electrophiles. We have now used in vivo genetic and in vitro biochemical methods to identify and demonstrate that Escherichia coli RclR (formerly YkgD) is a redox-regulated transcriptional activator of the AraC family, whose highly conserved cysteine residues are specifically sensitive to oxidation by RCS. Oxidation of these cysteines leads to strong, highly specific activation of expression of genes required for survival of RCS stress. These results demonstrate the existence of a widely conserved bacterial regulon devoted specifically to RCS resistance.

Reactive chlorine species (RCS), including hypochlorous acid (HOCl) and chloramines, are powerful antimicrobial oxidants capable of chlorinating and oxidizing a wide range of biomolecules (1–3). RCS, such as those present in bleach, are important disinfectants in human medical, industrial, and household settings (4, 5). Moreover, they are major bactericidal components of the oxidative burst of neutrophils (3, 6, 7) and are implicated in controlling bacterial colonization of epithelial surfaces (8–10). There is now also increasing evidence that production of RCS is a common strategy among diverse eukaryotes for controlling bacterial populations (11, 12). Understanding how bacteria sense and respond to RCS is therefore important for understanding the interactions between bacteria and their eukaryotic hosts, with obvious implications for the study of human health and disease.

Bacterial responses to reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) or superoxide (O₂⁻), have been studied extensively (reviewed recently in Refs. 13–16). It is known that these responses depend on select transcription factors (e.g. OxyR, SoxR, PerR), which are able to specifically sense particular oxidants, often through the oxidation state of conserved cysteine residues. These regulators control the expression of genes that contribute directly to ROS detoxification or to the repair of ROS-mediated damage. The ability of redox-sensitive regulators to distinguish among different oxidants is a key factor in redox signaling (17).

Recent studies have identified several bacterial transcription factors that respond to RCS treatment. These include the Escherichia coli transcription factors HypT and NemR and the Bacillus subtilis transcription factors OhrR and HypR (18–21). Interestingly, whereas HypT is so far only known to respond to HOCl (19, 22), the other RCS-sensing transcription factors respond to a variety of other stress signals, including cysteine-modifying electrophiles (20, 21, 23) and organic hydroperoxides (18, 24). In contrast to ROS and toxic electrophiles, which have a more limited set of cellular targets (25–27), RCS are capable of damaging proteins, DNA, lipids, and most other cellular components (1–3). It therefore appears that to successfully combat RCS, organisms require a multifaceted stress response system, regulated by a diverse set of redox-sensitive regulators.

Here we report the discovery of RclR (formerly known as YkgD), a highly RCS-specific transcriptional activator in E. coli. In vivo and in vitro studies demonstrate that RclR relies on the reversible oxidation of conserved redox-sensitive cysteine residues to control the expression of three genes essential for bac-
**RclR Is a Reactive Chlorine-specific Activator**

**EXPERIMENTAL PROCEDURES**

**Sequence Analysis and Primer Design**—Gene and protein sequences were obtained from the Integrated Microbial Genomes database (28). Custom Python scripts employing the Biopython 1.57 toolkit (29) were used to search for and sort RclR homologs from the National Center for Biotechnology Information databases. Sequence alignments were performed using MUSCLE 3.8 (30), and sequence logos were generated using WebLogo 3.1. Mutagenic primers were designed with PrimerX, qRT-PCR primers were designed with Primer3 0.4.0, and PCR and sequencing primers were designed with WebPrimer. All primers used in this study are listed in Table 1.

**Bacterial Strains and Growth Conditions**—All strains and plasmids used in this study are listed in Table 2. DNA manipulations were carried out by standard methods (31) in E. coli XL1 Blue (Stratagene), and the identity of all constructs was confirmed by sequencing (GENEWIZ, Inc.). E. coli was grown in lysogenic broth (LB; Fisher) (32) or MOPS minimal medium (Teknova, Inc.) containing 0.2% glucose, 1.32 mM K$_2$HPO$_4$, and 10 mm thiamine with ampicillin (100 µg mL$^{-1}$) or chloramphenicol (12.5 µg mL$^{-1}$) as indicated. Chemicals were from Fisher or Sigma-Aldrich. N-Chloroacetosyringone was synthesized before each use (33). Null mutations in E. coli MG1655 ($^{F_{-}}, \lambda^{+}, rph-1$lv$G^{-}$ rfb-50) (34) were constructed by replacement of genes with chloramphenicol resistance cassettes and subsequent resolution to yield in-frame deletions, as described previously (35), using the following primers (Table 1): rclR, [1] and [2]; rclA, [3] and [4]; rclB, [5] and [6]; rclC, [7] and [8].

**Plasmid Construction**—The rclR coding sequence plus 11 bp of 5’-sequence was amplified from E. coli MG1655 genomic DNA with primers [9] and [10] (Table 1) and cloned into the EcoRI and HindIII sites of plasmid pBAD30 (36) to yield plasmid pRCLR1. The QuikChange site-directed mutagenesis kit (Stratagene), modified to use only a single primer and 35 cycles of amplification, was used to mutate pRCLR1 with primers [11], [12], [13], or [14] to yield plasmids pRCLR8, pRCLR9, pRCLR14, and pRCLR15, containing rclR alleles encoding RclR$^{C21A}$, RclR$^{C89A}$, RclR$^{H42A}$, and RclR$^{H75A}$, respectively. The same method was used to mutate pRCLR8 with primer [12], yielding plasmid pRCLR12, containing an rclR allele encoding RclR$^{C21A,C89A}$.

For purification of RclR wild-type and mutant proteins, the rclR sequence was codon-optimized for expression in E. coli (GenScript, Inc.), yielding an rclR$^{opt}$ allele encoding the wild-type RclR amino acid sequence. The rclR$^{opt}$ allele was then cloned into the NdeI and BamHI sites of plasmid pET-15b (Novagen) to yield plasmid pRCLR7. The QuikChange site-directed mutagenesis kit (Stratagene), modified to use only a single primer and 35 cycles of amplification, was used to mutate pRCLR7 with primer [15], [16], [17], or [18] to yield plasmid pRCLR10, pRCLR11, pRCLR16, or pRCLR17. These plasmids contained rclR$^{opt}$ alleles encoding RclR$^{C21A}$, RclR$^{C89A}$, RclR$^{H42A}$, and RclR$^{H75A}$, respectively.

**HOCl Survival Assays**—HOCl survival assays were performed as described previously (20).

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**TABLE 1**

Primers used in this study

| Primer | Sequence |
|--------|----------|
| [1]    | 5’-TAC TAA GAT TGT TGG TGT TAT TAA TAA AAA ACC ACT CAG GAG TCT ACT ATG GTC GCT GCT CTG CTG C-3’ |
| [2]    | 5’-AAC TCC CCG AAT GGC GAT TAT CTT TCG CCT GAT TCC TGG CGG CTT TTA CAG AAT GAC GCG GCG TTA CAG AAA GAA GAC GCG GCT C-3’ |
| [3]    | 5’-CCG GCT TTA TGG TCG GCA CTC TCC TCG CTT GCT CTG C-3’ |
| [4]    | 5’-GAA TAA TCA GGG CGG GTT AGT CAT GTC GTG TAG CAC ACG TAT GAT A-3’ |
| [5]    | 5’-CCA CAA TAT TCA GCT CCG CTC CTT CAT GAT TAT CAA GAC GTC CAC ACG TAT GAT A-3’ |
| [6]    | 5’-GCA AGG CCT GCC TGA AAA GAA CTT CCG TGG TGG TGA C-3’ |
| [7]    | 5’-CAA CTT AGC TGG TTC CTC ACG CAC ACA TCA AAG GGG TGC AAG CTG TCT GA-3’ |
| [8]    | 5’-CAA ACA TTA GGG GCG GCT TGG TGA GAA GGC GCT ACG TAT GTC TCT ATG A-3’ |
| [9]    | 5’-TTC GGA TGC TAA GAG CTT GTA TCA TGG ATA TGT CAC ACG TAT GAT A-3’ |
| [10]   | 5’-TCC AGT TTT CCT ACT ACT ACT ACT ACT TTT TTA TCC TGG GGA TCT CTT CTT CAA TGG C-3’ |
| [11]   | 5’-CCA AAT TCA GCA GGC GCC TCT ACT GCC GCG GCG GCG TTA ACG CAA GGA GCG GCT C-3’ |
| [12]   | 5’-GAA TTA TCG GTT ATT CGT TGG GCG GCG TTA AGC CAA GGA GCG GCG C-3’ |
| [13]   | 5’-CCA CAA TAT TCA GCT CCG CTC CTT CAT GAT TAT CAA GAC GTC CAC ACG TAT GAT A-3’ |
| [14]   | 5’-GCA CTA TTA AGA TTA AAA ACC CCT GCT TGG TGG TGA C-3’ |
| [15]   | 5’-TCT ATT GAT CAT CTC GTC GGA GTC A-3’ |
| [16]   | 5’-TAT ATT GAT CAT CTC GTC GGA GTC A-3’ |
| [17]   | 5’-ACT GAG GCT ATT TAT TCT GCG GCC ACT GAC CCA GGG TGC A-3’ |
| [18]   | 5’-GCA ATG TTT CTA GGA AGA ACA GAA GAG GAC GCT C-3’ |
| [19]   | 5’-CAA AGA TAT TTG GCA GAT TAC TTT TAT GAT GAT A-3’ |
| [20]   | 5’-TAC TTA AGC TCT ACT ACT ACT ACT ACT TTT TTA TCC TGG GGA TCT CTT CTT CAA TGG C-3’ |
| [21]   | 5’-TCC AGT TTT CCT ACT ACT ACT ACT ACT TTT TTA TCC TGG GGA TCT CTT CTT CAA TGG C-3’ |
| [22]   | 5’-CCA AAT TCA GCA GGC GCC TCT ACT GCC GCG GCG GCG TTA ACG CAA GGA GCG GCT C-3’ |
| [23]   | 5’-GAA TTA TCG GTT ATT CGT TGG GCG GCG TTA AGC CAA GGA GCG GCG C-3’ |
| [24]   | 5’-CCA CAA TAT TCA GCT CCG CTC CTT CAT GAT TAT CAA GAC GTC CAC ACG TAT GAT A-3’ |
| [25]   | 5’-GCA CTA TTA AGA TTA AAA ACC CCT GCT TGG TGG TGA C-3’ |
| [26]   | 5’-TCT GCT TCA ACA CTT ACT ACT ACT ACT ACT TTT TTA TCC TGG GGA TCT CTT CTT CAA TGG C-3’ |
| [27]   | 5’-CTT TTC ATG ACC TGG AAC AGG CAA GAA GAG GAC GCT C-3’ |
| [28]   | 5’-ATG ACG CTC TCT ACT ACT ACT ACT ACT TTT TTA TCC TGG GGA TCT CTT CTT CAA TGG C-3’ |
| [29]   | 5’-ACT TCT GCT TTT ACT ACT ACT ACT ACT TTT TTA TCC TGG GGA TCT CTT CTT CAA TGG C-3’ |
| [30]   | 5’-GTT TTA TCT CTA GCG ACC TCA TAA TTT TTA TCC TGG GGA TCT CTT CTT CAA TGG C-3’ |
| [31]   | 5’-GTA AGA TCT CTT GCT CTA TGG CAC TGG ACG GAC GCT C-3’ |
| [32]   | 5’-ATC AGA AGT TTT CCT ACT ACT ACT ACT ACT TTT TTA TCC TGG GGA TCT CTT CTT CAA TGG C-3’ |
**RclR Is a Reactive Chlorine-specific Activator**

### Table 2

| Strains and plasmids used in this study | Relevant genotype | Source |
|----------------------------------------|------------------|--------|
| Plasmids                               |                  |        |
| pET-15b                                | Ap<sup>+</sup>   | Novagen(36) |
| pBAD30                                 | Ap<sup>+</sup>   |        |
| prCLR1                                 | Ap<sup>+</sup>   |        |
| prCLR7                                 | Ap<sup>+</sup>   |        |
| prCLR8                                 | Ap<sup>+</sup>   |        |
| prCLR9                                 | Ap<sup>+</sup>   |        |
| pBT10                                  | Ap<sup>+</sup>   |        |
| pC777                                  | Ap<sup>+</sup>   |        |
| pCR6                                   | Ap<sup>+</sup>   |        |
| pCR11                                  | Ap<sup>+</sup>   |        |
| pCR12                                  | Ap<sup>+</sup>   |        |
| pCR16                                  | Ap<sup>+</sup>   |        |
| pCR17                                  | Ap<sup>+</sup>   |        |

**Strains and plasmids used in this study**

Unless otherwise indicated, all strains and plasmids were generated in the course of this work. 

- **E. coli** Strains and Plasmids used in this study
  - **Plasmids**
    - pET-15b: N-terminal His<sub>6</sub> tag
    - pBAD30: P<sub>BAD</sub> arabinose-inducible promoter
    - prCLR1, prCLR7, prCLR8, prCLR9: P<sub>et</sub> promoter
    - pBT10: P<sub>BAD</sub> promoter
    - pC777: P<sub>C777</sub> promoter
    - pCR6: P<sub>CR6</sub> promoter
    - pCR11: P<sub>CR11</sub> promoter
    - pCR12: P<sub>CR12</sub> promoter
    - pCR16: P<sub>CR16</sub> promoter
    - pCR17: P<sub>CR17</sub> promoter

**Strains**

- XL1-Blue: Te<sup>+</sup>, Nal<sup>+</sup>
- BL21(DE3): F<sup>−</sup>, ompT gal dcm lon hsdSB (r<sup>−</sup> m<sup>−</sup>) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 amm7 nin5])
- MG1655: F<sup>−</sup>, λ, rph-1 ibvG- rfb-50
- MG1013: Cm<sup>+</sup>, F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 rclC::cat<sup>+</sup>
- MG1014: Cm<sup>+</sup>, F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 rclA::cat<sup>+</sup>
- MG1015: Cm<sup>+</sup>, F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 rclD::cat<sup>+</sup>
- MG1016: Cm<sup>+</sup>, F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 rclB::cat<sup>+</sup>
- MG403: F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 ∆nemR
- MG404: F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 ∆rclC
- MG406: F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 ∆rclD
- MG407: F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 rclR
- MG409: F<sup>−</sup>, λ, rph-1 ibvG- rfb-50 rclA

**Analysis of Cysteine Thiol Status**—Cysteine thiols in 8 µM reduced N-chlorotaurine-oxidized or H<sub>2</sub>O<sub>2</sub>-oxidized RclR were determined under denaturing conditions (50 mM sodium phosphate, 6 µM guanidinium HCl, 1 mM EDTA (pH 8.5)) using Ellman’s reagent (5,5’dithiobis(2-nitrobenzoic acid) (DTNB)) (38). For H<sub>2</sub>O<sub>2</sub>-oxidized samples, excess H<sub>2</sub>O<sub>2</sub> was removed by addition of 1 unit of bovine catalase (Sigma) and incubation at 37 °C for 15 min before addition of DTNB. Addition of catalase itself resulted in no detectable thiols under these conditions (data not shown).

The sulfenic acid-specific probes NBD-chloride and dione were used as described previously to examine reduced and oxidized RclR. P<sub>rclRA</sub> DNA (0.1 pmol) was incubated with 0–10.0 pmol of RclR protein in 10–µl reactions for 30 min at 37 °C, separated on 10% polyacrylamide gels in Tris borate/EDTA buffer for 90 min at 100 V, and stained with ethidium bromide; band intensity was quantified using ImageJ 1.44o (37).

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSA experiments were performed as described previously (20), with the following modifications. 

- **Gene Expression Analysis by qRT-PCR**—E. coli BL21(DE3) (Novagen). Cultures were grown in LB with shaking at 37 °C to an A<sub>600</sub> = 0.4–0.5. Then, potential RclR inducers were added as indicated. For plasmid-containing strains, media contained ampicillin and 1 mM arabinose. Samples (0.5 ml) were collected by centrifugation (1 min at 16,100 × g) immediately before and at indicated time points after addition of the respective stressors. RNA isolation and quantitative RT-PCR analyses were performed as described previously (20). Primers used in RT-PCRs were: 

- **Purification of RclR**—N-terminally His<sub>6</sub>-tagged RclR variants were overexpressed in E. coli BL21(DE3) (Novagen). Cultures were grown in LB with shaking at 37 °C to an A<sub>600</sub> = 0.6–0.8 and induced with 0.5 mM isopropyl-β-D-galactopyranoside, then incubated at 20°C for 20 h. Cultures were centrifuged at 6000 rpm for 20 min, resuspended in 0.5 M NaCl, 5 mM imidazole, 20 mM sodium phosphate (pH 7.4), and EDTA-free Complete Mini protease inhibitor (Roche Applied Science). Cells were lysed by three passages through a French press at 1200 psi. Lysates were spun down for 60 min at 20,000 rpm at 4°C, passed through a 0.8-µm filter, then applied to a nickel-loaded HiTrap Chelating HP affinity column (GE Healthcare). RclR was purified using an AKTA fast protein liquid chromatography system (Amersham Biosciences) with a 17-column volume gradient linear to 0.5 M NaCl, 0.5 mM imidazole, 20 mM sodium phosphate (pH 7.4). Fractions containing pure RclR were pooled and dialyzed against 50 mM sodium phosphate (pH 8.5), 0.5 M NaCl, 2 mM dithiothreitol (DTT), 10% glycerol and stored at −80°C.

**Analysis of Cysteine Thiol Status**—Cysteine thiols in 8 µM reduced N-chlorotaurine-oxidized or H<sub>2</sub>O<sub>2</sub>-oxidized RclR were determined under denaturing conditions (50 mM sodium phosphate, 6 µM guanidinium HCl, 1 mM EDTA (pH 8.5)) using Ellman’s reagent (5,5’dithiobis(2-nitrobenzoic acid) (DTNB)) (38). For H<sub>2</sub>O<sub>2</sub>-oxidized samples, excess H<sub>2</sub>O<sub>2</sub> was removed by addition of 1 unit of bovine catalase (Sigma) and incubation at 37°C for 15 min before addition of DTNB. Addition of catalase itself resulted in no detectable thiols under these conditions (data not shown).

The sulfenic acid-specific probes NBD-chloride and dione were used as described previously to examine reduced and oxidized RclR. P<sub>rclRA</sub> DNA (0.1 pmol) was incubated with 0–10.0 pmol of RclR protein in 10–µl reactions for 30 min at 37°C, separated on 10% polyacrylamide gels in Tris borate/EDTA buffer for 90 min at 100 V, and stained with ethidium bromide; band intensity was quantified using ImageJ 1.44o (37). Student’s t test was used to determine whether mean band intensity values differed significantly from each other. For reduction and re-reduction, samples were incubated in 1 mM DTT at 37°C for 15 min immediately before incubation with P<sub>rclRA</sub>. Metal-free reagents were prepared by incubation with 50 mg l<sup>−1</sup> Chelex 100 (Sigma) according to the manufacturer’s instructions.
N-chlorotaurine-oxidized RclR, RclRC21A, and RclRC89A (39, 40).

Testing the Role of Metals in RclR Activation—Metal content of purified wild-type RclR was analyzed using inductively coupled plasma-high resolution mass spectrometry at the Keck Elemental Geochemistry Laboratory, Department of Geologic Sciences (University of Michigan). To prepare metal-free protein samples, RclR (40 μM) was incubated with 1 mM EDTA, TPEN, 2,2′-dipyridyl, or EGTA at 37 °C for 1 h in metal-free buffer.

RESULTS

The RclR-regulated rcl locus is required for HOCl survival—Microarray analyses in a number of RCS-stressed E. coli strains revealed the massive up-regulation of three genes of unknown function ykgB, ykgl, and ykgC (20, 41, 42). All three genes are divergently transcribed from ykgD (Fig. 1A), encoding an uncharacterized AraC family transcriptional activator (43) whose expression was also significantly up-regulated after HOCl treatment. We therefore hypothesized that this locus might be involved in bacterial survival of HOCl stress and that ykgD might encode a HOCl-responsive transcription factor. Analysis of the HOCl sensitivity of in-frame deletion mutants lacking any one of the four ykg genes fully supported this assumption. All mutant strains were found to be more sensitive to HOCl treatment than wild-type cells (Fig. 1B), indicating that these genes are indeed involved in surviving HOCl stress. We therefore renamed the ykgD, ykgC, ykgl, and ykgB genes to rclR, rclA, rclB, and rclC, respectively, to reflect their role in reactive chlorine resistance (Fig. 1A). Quantitative RT-PCR analysis confirmed the microarray analysis and revealed that, upon sublethal HOCl stress, expression of rclA, rclB, and rclC was rapidly induced >100-fold, whereas expression of the putative regulator rclR increased 10-fold (Fig. 1C, upper panel). Expression levels remained high for at least 30 min after HOCl treatment. Induction of rclA, rclB, and rclC was largely eliminated in a ΔrclR mutant (Fig. 1C, lower panel). These results serve to demonstrate that rclR encodes a HOCl-sensitive transcriptional activator that controls the expression of rclA, rclB, and rclC and contributes significantly to bacterial HOCl survival.

RclR-dependent Gene Expression Is Specifically Activated by Reactive Chlorine Species—Of the few HOCl-sensitive transcription factors that have been identified to date, only HypT appears to be specific for RCS whereas all others respond to multiple stresses, including organic peroxides and electrophiles. To determine the specificity of RclR activation in vivo, we therefore treated wild-type E. coli with a variety of chlorinated compounds, reactive oxygen species, reactive nitrogen species, and electrophiles (Fig. 2). The chosen concentrations of each compound led to a short growth delay under our conditions, but did not result in cell death (data not shown). Addition
of HOCl or \(N\)-chlorotaurine, a reactive chloramine generated by the reaction of HOCl with taurine and found at high concentrations in neutrophils (7), resulted in 100–500-fold induction of \(rclB\) gene expression. No activation was seen with other, non-reactive chlorinated compounds, including chloroform and trichloroacetic acid. RclR also did not respond to oxidative stress caused by the organic hydroperoxide tert-butyl hydroperoxide, \(H_2O_2\), the superoxide-generating redox cycling agent methyl viologen (44), or by potassium tellurite (45). Similarly, no induction of \(rclB\) was observed after treatment with reactive nitrogen species, including the nitric oxide donor diethylamine nitric oxide and peroxynitrite, or with reactive electrophiles, including diamide, methylglyoxal, and \(N\)-ethylmaleimide. These results demonstrate that with RclR, we have identified a member of the AraC family that responds specifically to reactive chlorine species.

**Reactive Chlorine-specific Oxidation Activates RclR DNA Binding Activity**—To investigate the ability of RclR to bind and shift DNA in a potentially redox-dependent manner, we conducted EMSAs using purified RclR and the \(rclR-rclA\) intergenic region (\(P_{rclRA}\)) as a DNA substrate. Due to the remarkably high number of rare codons in the \(rclR\) coding sequence (i.e. one AGG, two AGA, and four CGG arginine codons, two ATA isoleucine codons, and one CCC proline codon) (46), which resulted in poor overexpression from the native gene sequence (data not shown), RclR was overexpressed for purification from an \(rclR\) gene that was codon-optimized for expression in \(E. coli\) (GenScript, Inc.). This strategy allowed high overexpression of RclR and the purification of the protein to >99% purity. EMSAs revealed that, whereas purified RclR did not bind to this DNA fragment under reducing conditions, it bound strongly to DNA after incubation with a 1:1 molar ratio of \(N\)-chlorotaurine (Fig. 3A). This activation was largely reversible by incubation of oxidized RclR with 2 mM of the thiol-specific reducing agent DTT, strongly suggesting that activation of RclR involves formation of reversible oxidative cysteine modifications. \(N\)-Chlorotaurine-activated RclR did not bind to a DNA fragment containing the \(nemR\) promoter region (20) (Fig. 3B), indicating that the DNA binding of RclR is sequence-specific and that RclR does not affect expression of the HOCl-responsive \(nemR\) operon. Confirming this result, qRT-PCR showed no changes in \(nemR\) expression in a \(\Delta rclR\) mutant strain or in \(rclB\) expression in a \(\Delta nemR\) mutant (data not shown), indicating that NemR and RclR control independent HOCl-responsive regulons in \(E. coli\).

Incubation of purified RclR with increasing molar ratios of \(H_2O_2\) resulted in some activation of DNA binding (Fig. 3C); but even at a ratio of 20:1, the activity of \(H_2O_2\)-treated RclR was significantly lower (Student’s \(t\) test, \(p < 0.05\)) than the activity of RclR treated with a 1:1 ratio of \(N\)-chlorotaurine to RclR (Fig. 3A). The \(in vivo\) sensitivity of RclR to \(H_2O_2\) was much lower than that reported for the \(bona fide\) \(H_2O_2\)-sensing activator OxyR, which is present in the oxidized, active form without the addition of \(H_2O_2\) in \(in vitro\) (47) and is fully activated by as little as 10 \(\mu M\) \(H_2O_2\) in \(in vivo\) (48). In contrast, 2 \(\mu M\) \(H_2O_2\) had no effect...
on RclR-dependent gene expression in vivo (Fig. 2). These results demonstrate that activation of RclR DNA binding activity is highly sensitive and specific to RCS and suggest that $H_2O_2$ is not a physiologically relevant activator of RclR. The fact that RclR activation is largely reversible in vitro by DTT strongly suggests that it is also reversible in vivo.

**Thiol Properties and Structural Characteristics of Oxidatively Activated RclR**—To further characterize the properties of oxidatively activated RclR, we next measured the cysteine thiol status in reduced, $N$-chlorotaurine-oxidized, or $H_2O_2$-oxidized RclR using Ellman’s assay (Fig. 4A). Of the 6 cysteine residues that are present in RclR, we detected an average of 5.5 reduced cysteines in reduced RclR compared with 3.6 and 5.0 reduced cysteines in RclR treated with a 1:1 molar ratio of $N$-chlorotaurine or a 10:1 molar ratio of $H_2O_2$, respectively. In combination with the findings presented in Fig. 3, this result suggests that activation of RclR by $N$-chlorotaurine may involve formation of one disulfide bond and explains the much reduced capacity of $H_2O_2$ to activate RclR. Nonreducing SDS-PAGE of reduced and oxidized RclR samples did not reveal the formation of higher molecular mass species upon oxidation (Fig. 4B), excluding the formation of intermolecularly disulfide-bonded oligomers.

$N$-Chlorotaurine treatment of cysteine is expected to result in the oxidation of the thiol (-SH) group to sulfenic acid (-SOH) (49). Reduced thiols and sulfenic acids can be detected using the probe NBD-chloride, which forms adducts with absorption maxima of 420 nm for thiols and 350 nm for sulfenic acid (39). However, no sulfenic acid adducts of NBD-chloride were detected in peroxide-treated GAPDH (40), no sulfenic acid was detected in RclR by this method either (data not shown). These results suggest that any sulfenic acid intermediates formed during the activation of RclR must exist only very transiently. The precise nature of the oxidative modification(s) in activated RclR remains to be elucidated.

**Mechanism of RclR Activation**—Based on RclR homology to regulators of the AraC family (43), RclR is predicted to be a two-domain protein with an N-terminal domain presumed to be involved in sensing (residues 1–162) and a C-terminal DNA-binding domain. To identify conserved residues that might be important for RclR function, we identified proteins with domains homologous to the RclR N-terminal domain (BLAST e-value < 0.0001) from 70 bacterial species. Alignment of these sequences (Fig. 5A) revealed the presence of two conserved cysteine residues (Cys-21 and Cys-89 of E. coli RclR) and two conserved histidine residues (His-42 and His-75 of E. coli RclR). We also noted that the RclR N-terminal domain appears to be a member of the poorly characterized Cupin_6 (pfam12852) protein family (50). Cupins are a functionally diverse protein superfamily sharing a common thermostable small ß-barrel fold (51). However, very little is known about the functions or detailed structure of the Cupin_6 subfamily. Fig. 5B shows an alignment of the E. coli RclR sequence with the Cupin_6 consensus sequence, showing that Cys-89, His-42, and His-75 are conserved in both RclR and the larger Cupin_6 family, but that Cys-21 appears to be specific to RclR.

**RclR Does Not Appear to Require Metals for Activation**—The presence of two conserved cysteines and two conserved histidines in RclR, along with the known presence of biologically important metal ions in many cupin proteins (51), led us to hypothesize that RclR might be a metalloprotein. Bound metal ions can have dramatic effects on the redox properties of cysteine residues (52), and 2Cys-2His motifs are very common among zinc-binding proteins (53). However, inductively coupled plasma mass spectrometry failed to identify any stoichiometric metals bound to purified, redox-active RclR. To test further whether the presence of metals had any effect on activation of RclR, we treated purified RclR in metal-free buffers with a variety of metal-binding chelators. As shown in Fig. 6, extended treatment of RclR with high concentrations of either the strong zinc-binding chelator TPEN ($K_d$ for Zn$^{2+}$ = 10$^{-16}$ M$^{-1}$) (54) or with the general chelator EDTA had no significant effect on the activation of RclR in vitro (Student’s t test, p > 0.3). Similar results were observed with other chelators, including EGTA and 2,2-dipyridyl (data not shown). These results led us to conclude that, at least in vitro, RclR does not require metal ions for activation by $N$-chlorotaurine.

**Role of Conserved Cysteine and Histidine Residues in the Activation of RclR**—To first determine what role the conserved cysteines Cys-21 and Cys-89 play in redox sensing by RclR, we constructed plasmids encoding RclR variants in which one or both conserved cysteines were mutated to alanine. The ability whether the presence of metals had any effect on activation of RclR, we treated purified RclR in metal-free buffers with a variety of metal-binding chelators. As shown in Fig. 6, extended treatment of RclR with high concentrations of either the strong zinc-binding chelator TPEN ($K_d$ for Zn$^{2+}$ = 10$^{-16}$ M$^{-1}$) (54) or with the general chelator EDTA had no significant effect on the activation of RclR in vitro (Student’s t test, p > 0.3). Similar results were observed with other chelators, including EGTA and 2,2-dipyridyl (data not shown). These results led us to conclude that, at least in vitro, RclR does not require metal ions for activation by $N$-chlorotaurine.
**FIGURE 5.** Conserved residues in RclR.

A, alignment of RclR homologs from 70 bacterial species, with cysteine residues indicated in red and histidine residues in blue. Colored numbers in brackets indicate the E. coli numbering of conserved cysteine and histidine residues. B, alignment of E. coli RclR with the Cupin_6 domain consensus sequence (50), with conserved cysteine and histidine residues indicated with black highlighting. Asterisk indicates cysteine conserved among RclR homologs but not in the Cupin_6 consensus. Colored numbers in brackets indicate the E. coli numbering of conserved cysteine and histidine residues.

**FIGURE 6.** RclR does not require metals for activation in vitro. RclR was prepared by treatment under metal-free conditions for 1 h at 37 °C with the indicated chelators (1 mM), then assayed by EMSA for binding to PRclRA before and after treatment with a 1:1 molar ratio of N-chlorotaurine. Representative gels are shown, along with quantification results (mean ± S.D. (error bars)).
RCS-response in vivo, the RclR-specific Cys-21 appears to play the more critical role in the RclR redox response. Next, we used the same strategy to determine the roles of the conserved histidines His-42 and His-75 in RCS sensing both in vivo and in vitro. In vivo, mutation of either histidine residue to alanine resulted in substantially higher rclB expression in the absence of HOCl, along with a slight further increase after HOCl treatment (Fig. 7). These results suggest that both conserved His residues are involved in preventing DNA binding when RclR is reduced. In vitro EMSAs agreed with these results and showed increased DNA binding by RclR<sup>H75A</sup> under reducing conditions compared with the wild type (Fig. 9). Binding of the RclR<sup>H42A</sup> and RclR<sup>H75A</sup> variants after N-chlorotaurine treatment (Fig. 9) was similar to that of the wild type, indicating that the lack of these histidines did not impair the activation of RclR by N-chlorotaurine. Interestingly, however, in vitro DNA binding of both RclR<sup>H42A</sup> and RclR<sup>H75A</sup> was activated more strongly by a 10:1 excess of H<sub>2</sub>O<sub>2</sub> than was the wild type (Fig. 9). Indeed, the RclR<sup>H42A</sup> variant was significantly more sensitive to oxidation by both N-chlorotaurine and H<sub>2</sub>O<sub>2</sub> than the wild type (Student’s t test, p < 0.05). In combination, these results suggested that His-42 and His-75 play roles in maintaining RclR in an inactive state under reducing conditions and contribute, by a yet to be defined mechanism, to the RCS specificity and oxidation sensitivity of RclR.

DISCUSSION

We have now identified RclR as a transcriptional activator that relies on conserved redox-sensitive cysteine residues to specifically sense RCS and control the expression of genes that contribute to the ability of E. coli to survive HOCl stress. The strong in vivo RCS specificity of RclR distinguishes it from most other HOCl-responsive transcription factors so far described. For example, E. coli NemR and B. subtilis HypR respond to both HOCl and cysteine-modifying electrophiles (e.g. N-ethylmaleimide or diamide) (20, 21), and B. subtilis OhrR responds to both HOCl and organic hydroperoxides (18, 55, 56). Our results suggest a model in which initial oxidation of Cys-21 leads to partial activation of RclR, followed by the formation of an intramolecular disulfide bond between Cys-21 and Cys-89, which stabilizes the fully active form of the protein in vivo. Cys-21 is necessary for activation and is likely to be the critical redox-sensitive cysteine of RclR. The ability of the RclR<sup>C89A</sup> variant to be activated in vitro supports this conclusion and demonstrates that disulfide bond formation is indeed not required for RclR activation in vitro. As shown in Fig. 8, the C89A variant can in fact be fully activated in vitro. Formation of a Cys-21–Cys-89 disulfide bond might serve to stabilize the active form of RclR under physiological redox conditions in vivo and might prevent premature reduction and inactivation by cellular reductases (e.g. thioredoxin or glutaredoxin). Regardless, in vivo, both con-

![Figure 7](image_url1)

![Figure 8](image_url2)
served cysteines are required for sensing of RCS. This model resembles the mechanism of redox activation of OxyR, in which initial oxidation of Cys-199 precedes formation of a Cys-199–Cys-208 disulfide bond in the fully activated protein (57). Although no structural information for RclR or any other Cupin_6 family member is available, modeling of the RclR N-terminal domain with the I-TASSER (58) and Swiss-Model (59) structure prediction algorithms supports this model. Despite substantial differences in the structures predicted by these two algorithms, they agree in placing Cys-21 and Cys-89 adjacent to each other on adjoining β strands (data not shown), well positioned to form a disulfide bond.

RclR is unusual not only for its high specificity and sensitivity to RCS, but also for being a redox-sensitive transcriptional activator. Most known redox-sensing transcription factors function as repressors and are inactivated by oxidation (e.g. NemR, OhrR, PerR, and HypR) (20, 21, 24, 60). Inactivation then leads to their release from DNA and to the de-repression of gene expression. RclR now joins the relatively small group of regulators whose functions are specifically activated by oxidation (e.g. OxyR and SoxR) (13, 14). In contrast to the activation of DNA binding observed in RclR, however, OxyR and SoxR bind to DNA in both their reduced and oxidized forms, with oxidation resulting in structural changes that lead to transcriptional activation (47, 61).

The mechanism underlying RclR specificity for RCS remains to be determined, but our results demonstrate that it differs from that of other known RCS-specific proteins: the chaperone Hsp33 relies on the presence of bound zinc to modulate the redox sensitivity of its cysteine residues (52, 62), whereas HypT, the only other RCS-specific transcription factor so far described, relies on HOCl-dependent methionine oxidation (19, 22). The mechanism of RclR activation also appears to be distinct from that of other known HOCl-responsive transcription factors, including NemR and HypR, whose DNA binding activity is inactivated by the formation of disulfide-bonded oligomers (20, 21) or OhrR, whose inactivation depends on S-bacillithiolation or sulfinamide formation at a conserved cysteine residue (18, 55).

We are currently pursuing structural analysis of RclR to identify both the specific nature of the structural changes associated with oxidative activation and properties of Cys-21 or Cys-89 which may contribute to the exquisite sensitivity of RclR to RCS.

An intriguing unanswered question is why bacteria appear to have evolved multiple overlapping RCS-sensitive regulons as opposed to the simpler regulatory architecture devoted to resisting other oxidative stress conditions such as peroxide or superoxide. E. coli contains one transcription factor devoted to sensing H$_2$O$_2$ (OxyR) and one devoted to sensing superoxide (SoxR) (13). There is now evidence that E. coli possesses at least three different regulators that respond very sensitively to HOCl (RclR, HypT, and NemR) and which control distinct, nonoverlapping stress responses (19, 20). It remains to be determined what properties of RCS stress require such a complex transcriptional response.
The function of RclR-regulated genes and how their products contribute to survival of HOCl stress are also currently unknown. RclA is a group I flavin disulfide reductase (63), predicted to be very similar to thioredoxin reductase and mercuric reductase. RclB is an 80-amino acid periplasmic protein, and RclC is a transmembrane protein with some homology to quinone-binding proteins. It is tempting to hypothesize that these proteins form a membrane-associated complex responsible for reducing cellular components specifically oxidized by RCS. RclR homologs are found in a variety of α-, β-, γ-, and δ-proteobacteria as well as in a few actinobacteria. Among the Enterobacteriaceae, the E. coli-like rel locus architecture is conserved. However, in other clades the rclR homologs are found associated with genes other than rclA, rclB, and rclC. The most commonly found rclR-associated genes encode a homolog of the AhpD alkyl hydroperoxidase (64), an oxidative stress resistance enzyme which we hypothesize may play a so far uncharacterized role in RCS resistance in these organisms.

In conclusion, our studies demonstrate that RclR is a conserved transcriptional activator that depends on the oxidation of conserved cysteine residues to respond very sensitively and specifically to RCS. In E. coli, RclR regulates the high level expression of rclA, rclB, and rclC, genes that play important roles in surviving RCS treatment.

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RclR Is a Reactive Chlorine-specific Activator

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