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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),[3] 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 (H_2O_2) or superoxide ($O_2^{\cdot-}$), 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-

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[3] The abbreviations used are: RCS, reactive chlorine species; HOCl, hypochlorous acid; qRT-PCR, quantitative RT-PCR; ROS, reactive oxygen species; TPEN, tetrakis-(2-pyridylmethyl)ethylenediamine; NBD-chloride, 4-chloro-7-nitro-1,2,3-benzoxadiazole.

TABLE 1
Primers used in this study

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

terial survival of reactive chlorine stress (*rclA*, *rclB*, and *rclC*; formerly *ykgC*, *ykgI*, and *ykgB*, respectively). Deletion of any one of these genes, whose RCS-mediated transcriptional upregulation is among the most pronounced in *E. coli*, dramatically increases RCS stress sensitivity. These results suggest that we have identified an important member of the RCS stress response system in bacteria.

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 Web Primer. 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_2HPO_4, and 10 μM thiamine with ampicillin (100 μg ml^{-1}) or chloramphenicol (12.5 μg ml^{-1}) as indicated. Chemicals were from Fisher or Sigma-Aldrich. *N*-Chlorotaurine was synthesized before each use (33). Null mutations in *E. coli* MG1655 (F^-, λ^-, *rph-1 ilvG*$^-$ *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).

TABLE 2

Strains and plasmids used in this study

Unless otherwise indicated, all strains and plasmids were generated in the course of this work. Tc^R, tetracycline resistance; Nx^R, nalidixic acid resistance; Cm^R, chloramphenicol resistance; Ap^R, ampicillin resistance.

Strain name	Marker(s)	Relevant genotype	Source
***E. coli* strains**			
XL1-Blue	Tc^R Nx^R	*endA1 gyrA96*(nal^R) *thi-1 recA1 relA1 lac glnV44* F'[::Tn10(*tet*$^+$) *proAB*$^+$ *lacI*$_q$ Δ(*lacZ*)M15] *hsdR17*(r_K^- m_K^+)	Stratagene
BL21(DE3)		F$^-$, *ompT gal dcm lon hsdSB* (r_B^- m_B^-) λ (DE3 [*lacI lacUV5*-T7 gene 1 *ind1 sam7 nin5*])	Novagen
MG1655		F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50*	(34)
MJG013	Cm^R	F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50 rclC*::*cat*$^+$	
MJG014	Cm^R	F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50 rclA*::*cat*$^+$	
MJG015	Cm^R	F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50 rclR*::*cat*$^+$	
MJG016	Cm^R	F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50 rclB*::*cat*$^+$	
MJG043		F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50* Δ*nemR*	(20)
MJG045		F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50* Δ*rclC*	
MJG046		F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50* Δ*rclA*	
MJG047		F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50* Δ*rclB*	
MJG059		F$^-$, λ$^-$, *rph-1 ilvG*$^-$ *rfb-50* Δ*rclR*	
Plasmids			
pET-15b	Ap^R	N-terminal His_6 tag	Novagen
pBAD30	Ap^R	P_{BAD} arabinose-inducible promoter	(36)
pRCLR1	Ap^R	*rclR*$^+$	
pRCLR7	Ap^R	*rclR*$^{opt.}$	
pRCLR8	Ap^R	*rclR*^{T61G,G62C,C63G} (encoding $RclR^{C21A}$)	
pRCLR9	Ap^R	*rclR*^{T265G,G266C,C267G} (encoding $RclR^{C89A}$)	
pRCLR10	Ap^R	*rclR*$^{opt.(T61G,G62C,T63G)}$ (encoding $RclR^{C21A}$)	
pRCLR11	Ap^R	*rclR*$^{opt.(T265G,G266C,T267G)}$ (encoding $RclR^{C89A}$)	
pRCLR12	Ap^R	*rclR*^{T61G,G62C,C63G,T265G,G266C,C267G} (encoding $RclR^{C21A,C89A}$)	
pRCLR16	Ap^R	*rclR*$^{opt.(C124G,\ A125C,C126G)}$ (encoding $RclR^{H42A}$)	
pRCLR17	Ap^R	*rclR*$^{opt.(C223G,A224C,T225G)}$ (encoding $RclR^{H75A}$)	

Gene Expression Analysis by qRT-PCR—E. coli was grown in MOPS medium with shaking at 37 °C to an A_{600} = 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: *rrsD*, [19] and [20]; *rclR*, [21] and [22]; *rclA*, [23] and [24]; *rclB*, [25] and [26]; *rclC*, [27] and [28]; *nemR*, [29] and [30]. Student's *t* test was used to determine whether the means of log-transformed expression values differed significantly from each other.

Purification of RclR—N-terminally His_6-tagged RclR variants were overexpressed in *E. coli* BL21(DE3) (Novagen). Cultures were grown in LB with shaking at 37 °C to an A_{600} = 0.6–0.8 and induced with 0.5 mM isopropyl 1-thio-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) plus 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 ÄKTA fast protein liquid chromatography system (Amersham Biosciences) with a 17-column volume linear gradient to 0.5 M NaCl, 0.5 M 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.

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed as described previously (20), with the following modifications. P_{rclRA}, the 225-bp intergenic region between *rclA* and *rclR*, was amplified using primers [31] and [32]. P_{nemR} was prepared as described previously (20). RclR protein variants were exchanged into 150 mM NaCl, 50 mM sodium phosphate (pH 6.8) using Bio-Spin P-30 (Bio-Rad) gel filtration columns and centrifuged for 30 min at 16,000 × *g* at 4 °C to remove aggregates. RclR proteins were then incubated with the indicated oxidants at 37 °C for 15 min. To remove the oxidants, RclR was applied to a Bio-Spin P-30 column, equilibrated with 150 mM NaCl, 50 mM sodium phosphate, 10% glycerol (pH 6.8) and centrifuged for 30 min at 16,000 × *g* at 4 °C. P_{rclRA} 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_{rclRA}. Metal-free reagents were prepared by incubation with 50 mg l^{-1} Chelex 100 (Sigma) according to the manufacturer's instructions.

Analysis of Cysteine Thiol Status—Cysteine thiols in 8 μM reduced *N*-chlorotaurine-oxidized or H_2O_2-oxidized RclR were determined under denaturing conditions (50 mM sodium phosphate, 6 M guanidinium HCl, 1 mM EDTA (pH 8.5)) using Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)) (38). For H_2O_2-oxidized samples, excess H_2O_2 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 dimedone were used as described previously to examine reduced and

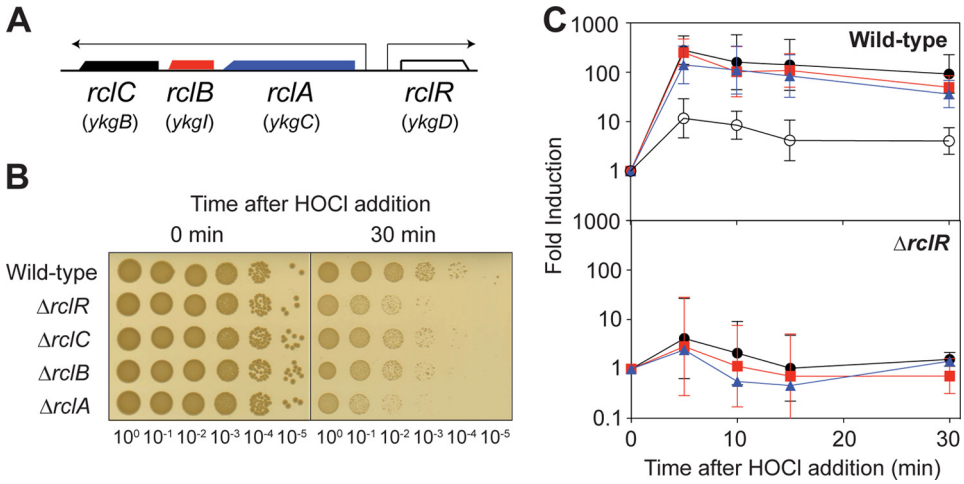

FIGURE 1. **The RclR-regulated *rcl* locus is required for HOCl survival.** *A*, operon structure of the *rcl* locus shows the former names of the *rcl* genes in *parentheses*. *B, E. coli* MG1655 (wild type) and isogenic mutant strains were incubated in MOPS glucose medium containing 2.5 mM HOCl for 30 min, then diluted and spot-titered onto LB agar. Plates were incubated at 37 °C overnight. *C, E. coli* MG1655 (wild type) and the Δ*rclR* mutant were grown to mid-log phase in MOPS glucose medium and treated with 0.4 mM HOCl. Expression ratios of *rclR* (*open circles*), *rclA* (*blue triangles*), *rclB* (*red squares*), or *rclC* (*black circles*) were determined by qRT-PCR (mean ± S.D. (*error bars*)).

N-chlorotaurine-oxidized RclR, RclR^{C21A}, and RclR^{C89A} (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 Geological 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*, *ykgI*, and *ykgC* (20, 41, 42). All three genes are divergently transcribed from *ykgD* (Fig. 1*A*), 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 agreed with this assumption. All mutant strains were found to be more sensitive to HOCl treatment than wild-type cells (Fig. 1*B*), indicating that these genes are indeed involved in surviving HOCl stress. We therefore renamed the *ykgD*, *ykgC*, *ykgI*, and *ykgB* genes to *rclR*, *rclA*, *rclB*, and *rclC*, respectively, to reflect their role in reactive chlorine resistance (Fig. 1*A*). 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. 1*C*, *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 Δ*rclR* mutant (Fig. 1*C*, *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.

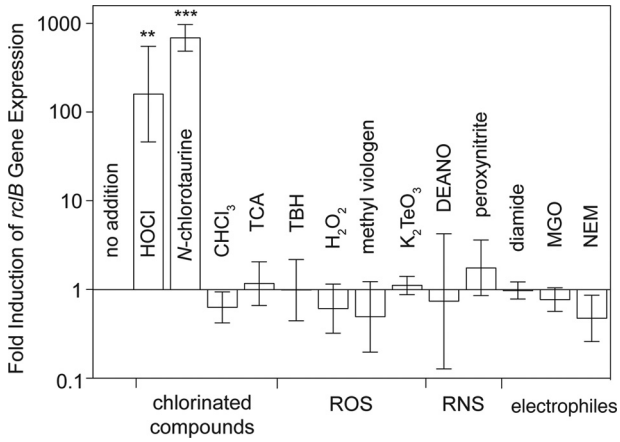

FIGURE 2. **RclR is specifically activated by reactive chlorine species *in vivo*.** *E. coli* MG1655 was grown to mid-log phase in MOPS glucose medium and treated with chlorinated compounds (0.4 mM HOCl, 0.1 mM *N*-chlorotaurine, 1% chloroform ($CHCl_3$), or 0.1% trichloroacetic acid (*TCA*)), ROS (0.25 mM *tert*-butyl hydroperoxide (*TBH*), 2 mM H_2O_2, 0.4 mM methyl viologen, or 2 μg ml^{-1} potassium tellurite (K_2TeO_3)), reactive nitrogen species (*RNS*) (0.2 mM diethylamine nitric oxide (*DEANO*) or 0.1 mM peroxynitrite), or electrophiles (0.5 μM diamide, 0.2 mM methylglyoxal (*MGO*), or 0.1 mM *N*-ethylmaleimide (*NEM*)). -Fold induction of *rclB* after 10 min was determined by qRT-PCR (mean ± S.D. (*error bars*)). Expression values indicated with *asterisks* are significantly different from expression in the absence of treatment (Student's *t* test; **, $p < 0.01$; ***, $p < 0.001$).

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

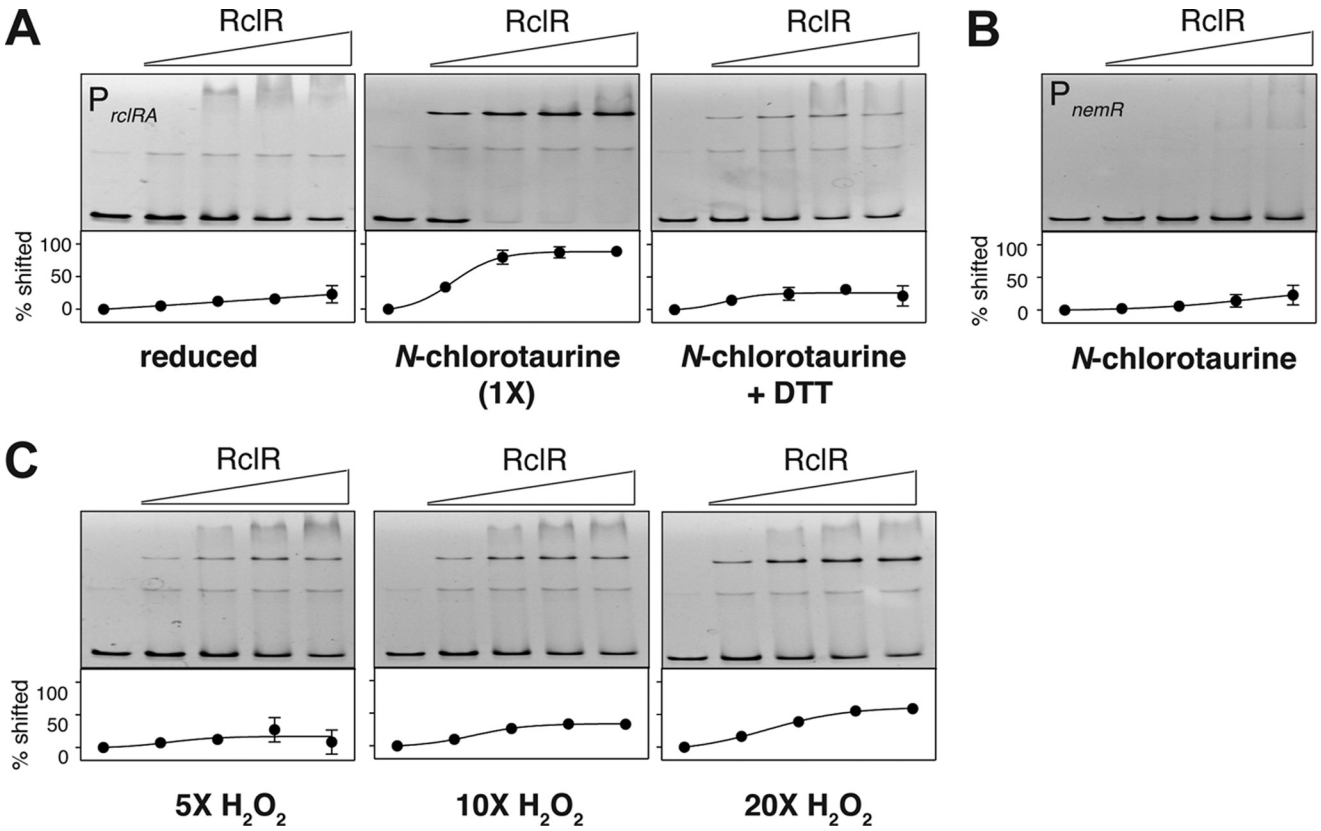

FIGURE 3. **Reactive chlorine-specific oxidation activates RclR DNA binding activity *in vitro*.** *A*, EMSAs of the binding of RclR (0, 0.25, 0.5, 0.75, 1 μM) to *rclRA* promoter DNA (P_{rclRA}, 10 nM) before and after treatment with a 1:1 molar ratio of *N*-chlorotaurine. To test the reversibility of oxidative activation, *N*-chlorotaurine-oxidized RclR was incubated for 30 min with the thiol-reducing agent DTT (1 mM). Representative gels are shown, along with quantification results (mean ± S.D. (*error bars*)). *B*, EMSA of *N*-chlorotaurine-oxidized RclR binding to *nemR* promoter DNA (P_{nemR}). *C*, EMSAs of RclR binding to P_{rclRA} after treatment with 5:1, 10:1, or 20:1 molar ratios of H_2O_2.

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, nonreactive 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. 3*A*). 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. 3*B*), 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 Δ*rclR* mutant strain or in *rclB* expression in a Δ*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. 3*C*); 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. 3*A*). The *in vitro* 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 vitro* (47) and is fully activated by as little as 10 μM H_2O_2 *in vivo* (48). In contrast, 2 mM H_2O_2 had no effect

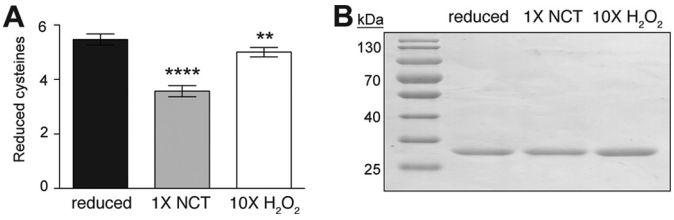

FIGURE 4. **Characteristics of oxidatively activated RclR.** *A*, cysteine thiol status of RclR, as determined by Ellman's assay, before and after treatment with a 1:1 molar ratio of *N*-chlorotaurine (*NCT*) or a 10:1 molar ratio of H_2O_2. *Asterisks* indicate significant differences from the untreated sample (Student's *t* test; **, $p < 0.01$; ****, $p < 10^{-8}$). *B*, nonreducing SDS-PAGE of RclR (2 μg) before and after treatment with a 1:1 molar ratio of *N*-chlorotaurine or a 10:1 molar ratio of H_2O_2.

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. 4*A*). 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. 4*B*), 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 *N*-chlorotaurine-oxidized RclR (data not shown). To confirm this result, *N*-chlorotaurine-oxidized RclR was treated with the sulfenic acid-specific nucleophile dimedone and examined using antibodies specific to the resulting thioether product (40). Whereas sulfenic acid formation was clearly 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. 5*A*) 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 β-barrel fold (51). However, very little is known about the functions or detailed structure of the Cupin_6 subfamily. Fig. 5*B* 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 of these mutants to respond to RCS was then tested both *in vivo* and *in vitro*. *In vivo*, qRT-PCR analysis (Fig. 7) showed that whereas *E. coli* strains encoding the wild-type RclR on a plasmid had higher base-line levels of *rclB* expression than did a strain encoding RclR on the chromosome (probably due to increased *rclR* copy number), *rclB* expression was strongly further induced by the addition of 0.4 mM HOCl. In contrast, strains encoding RclR variants lacking either Cys-21, Cys-89, or both Cys-21 and Cys-89 were unable to induce *rclB* expression in response to HOCl, indicating that both Cys-21 and Cys-89 play important roles in the activation of RclR *in vivo*. *In vitro* EMSA using the purified mutant RclR variants revealed that whereas the $RclR^{C21A}$ variant was no longer able to respond to *N*-chlorotaurine treatment by activation of DNA binding (Fig. 8, *middle panels*), the $RclR^{C89A}$ mutant variant maintained its redox response (Fig. 8, *bottom panels*). These results showed that, whereas both Cys-21 and Cys-89 residues are required for

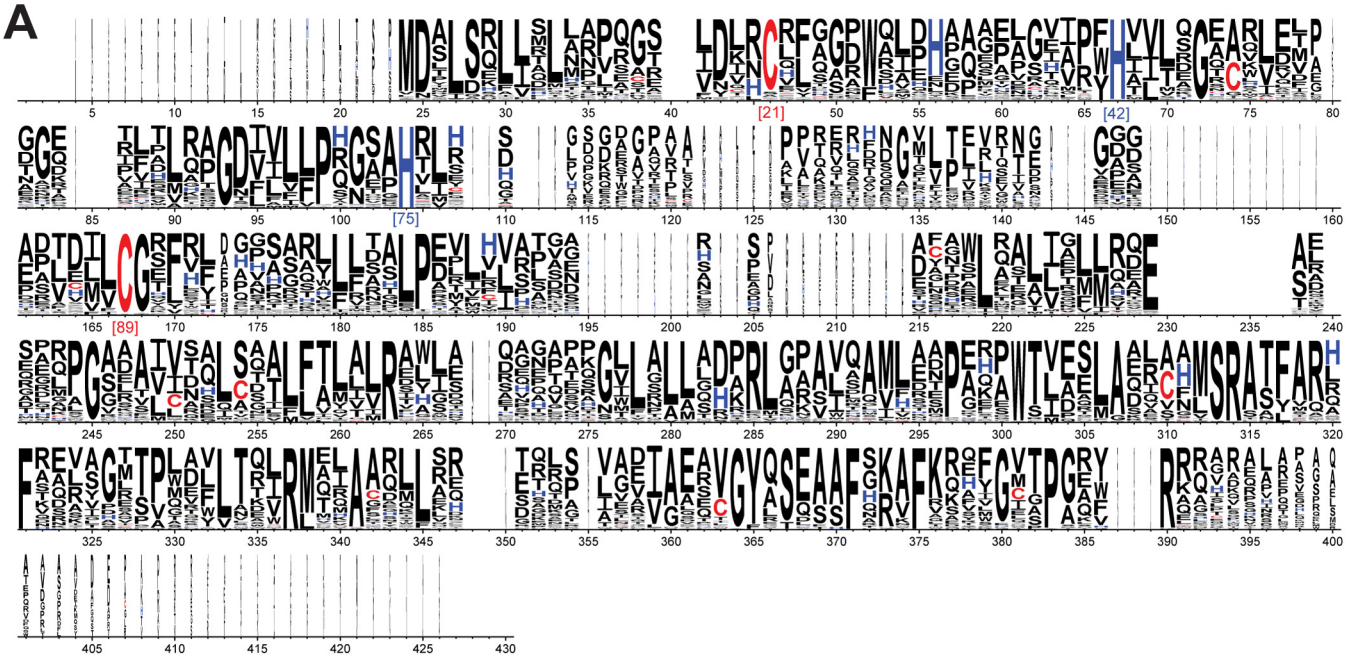

FIGURE 5. **Conserved residues in RclR.** *A*, alignment of RclR homologs from 70 bacterial species, with cysteine residues indicated in *red* and histidine residues in *blue*. *Colored numbers* in *brackets* indicate the *E. coli* numbering of conserved cysteine and histidine residues. *B*, alignment 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.

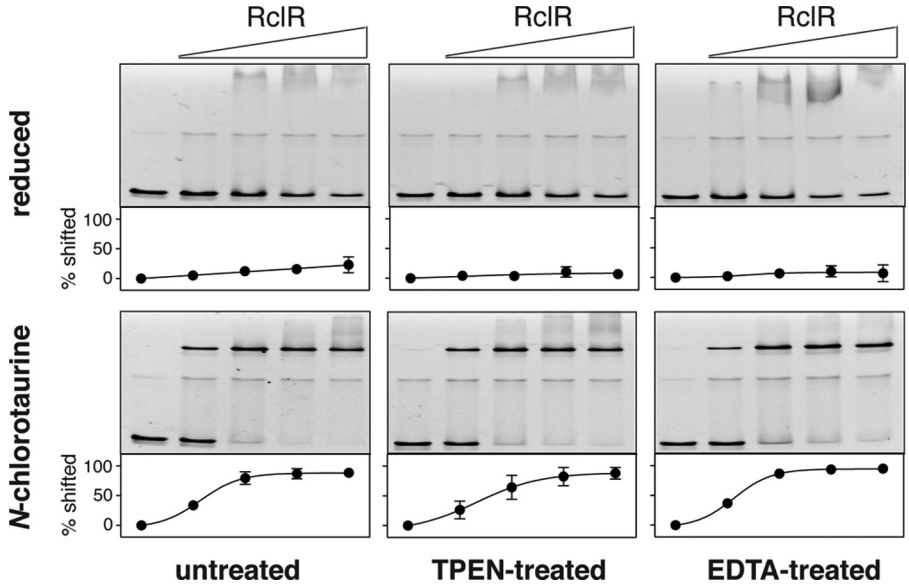

FIGURE 6. **RclR does not require metals for activation *in vitro*.** RclR was prepared by treatment under metal-free conditions for 1 h at 37 °C with the indicated chelators (1 mM), then assayed by EMSA for binding to P_{rclRA} before and after treatment with a 1:1 molar ratio of *N*-chlorotaurine. Representative gels are shown, along with quantification results (mean ± S.D. (*error bars*)).

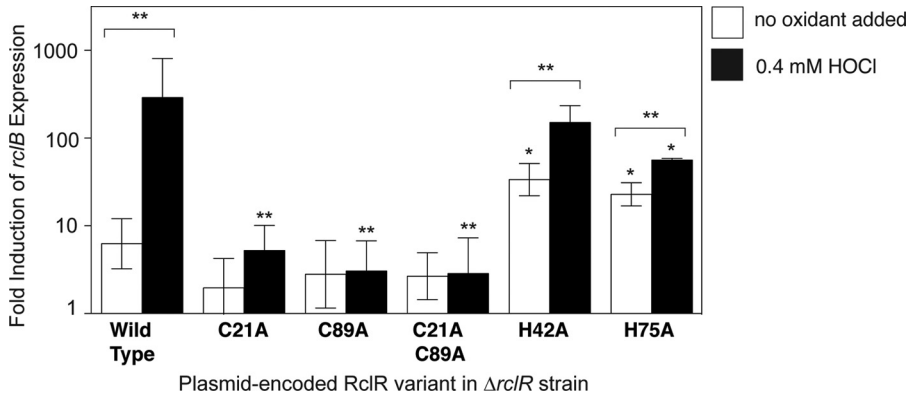

FIGURE 7. **Role of conserved cysteine and histidine residues in the activation of RclR *in vivo*.** *E. coli* Δ*rclR* strains containing pBAD30-derived plasmids encoding the indicated RclR variants were grown to mid-log phase in MOPS glucose medium and treated with 0.4 mM HOCl. -Fold induction of *rclB* after 10 min, relative to expression in untreated wild-type *E. coli* MG1655, was determined by qRT-PCR (mean ± S.D. (*error bars*)). Expression values indicated with *asterisks* directly above the *bars* are significantly different from expression under the same condition in the wild-type, whereas *asterisks* above *brackets* indicate RclR variants leading to significantly different expression with and without HOCl treatment (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$).

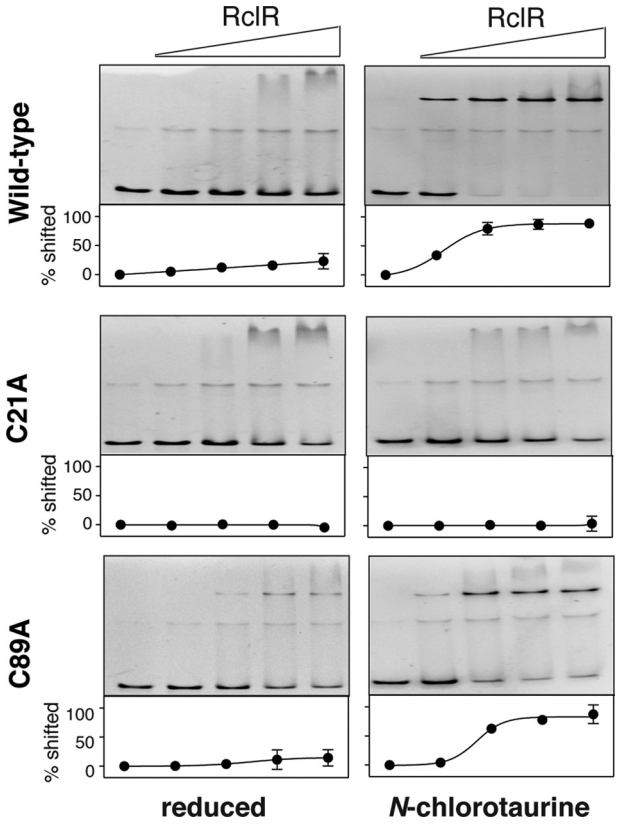

FIGURE 8. **Role of conserved cysteine residues in the activation of RclR *in vitro*.** EMSAs show binding of RclR wild-type, $RclR^{C21A}$, and $RclR^{C89A}$ variant proteins to P_{rclRA} before and after treatment with a 1:1 molar ratio of *N*-chlorotaurine. Representative gels are shown, along with 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 $RclR^{H75A}$ under reducing conditions compared with the wild type (Fig. 9). Binding of the $RclR^{H42A}$ and $RclR^{H75A}$ 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 $RclR^{H42A}$ and $RclR^{H75A}$ was activated more strongly by a 10:1 excess of H_2O_2 than was the wild type (Fig. 9). Indeed, the $RclR^{H42A}$ variant was significantly more sensitive to oxidation by both *N*-chlorotaurine and H_2O_2 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 $RclR^{C89A}$ 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-

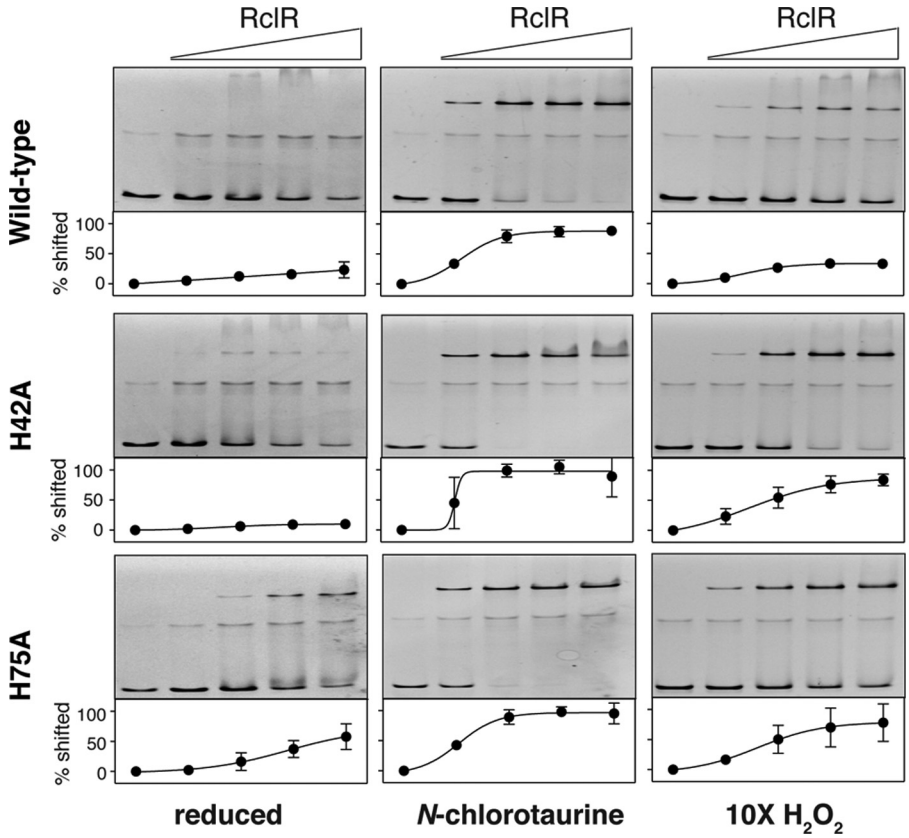

FIGURE 9. **Role of conserved histidine residues in the activation of RclR *in vitro*.** EMSAs show binding of RclR wild-type, $RclR^{H42A}$, and $RclR^{H75A}$ variant proteins to P_{rclRA} before and after treatment with a 1:1 molar ratio of *N*-chlorotaurine or a 10:1 molar ratio of H_2O_2. Representative gels are shown, along with quantification results (mean ± S.D. (*error bars*)).

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 sulfenamide 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_2O_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 *rcl* 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 gene encodes 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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