Hypochlorous acid (HOCl) is a powerful oxidant and one of the most commonly used disinfectants in the world (1). It is also naturally generated during the microbial oxidative burst of neutrophils and appears to play a key role in controlling bacterial colonization of mucosal epithelia (2, 3). Despite this physiological importance, little is known about how bacteria sense or defend themselves against reactive chlorine species (RCS), which include HOCl, chloramines, and other related compounds that are able to chlorinate and oxidize biomolecules (4).

Very few transcription factors sensitive to HOCl have been previously thought to be specific for sensing organic hydroperoxides, has been reported to be involved in HOCl resistance as well (6). Relatively little is known, however, about how these regulators and the genes they control contribute to HOCl survival. This is in contrast to the very large body of research that has been conducted to investigate how bacteria sense and defend themselves against other, less reactive bactericidal oxidants (4, 7) such as hydrogen peroxide (H₂O₂) or superoxide (O₂⁻) (for recent review, see Refs. 8–11).

Given the importance of HOCl in health and disease, we therefore decided to perform a more detailed investigation of how bacteria sense and respond to bleach. Here, we report our discovery that the widely conserved TetR family repressor NemR functions as a HOCl-responsive transcription factor in E. coli in addition to its previously described role in responding to cysteine-modifying electrophiles (12). In vitro and in vivo studies reveal that HOCl sensitivity is conferred upon NemR by cysteine residues that are highly sensitive toward oxidation by HOCl and related physiologically important RCS, such as N-chlorotaurine, an antimicrobial compound formed when HOCl reacts with taurine (2-aminoethanesulfonic acid) during the oxidative burst of neutrophils (13). RCS oxidation of cysteine residues, which is a fully reversible process in vitro and likely also in vivo, leads to a decrease in NemR DNA binding affinity, causing the derepression of downstream target gene expression. We demonstrate that bleach-mediated up-regulation of the NemR-controlled genes gloA and nemA increases bacterial bleach survival, providing the first evidence that resistance toward bleach relies on the ability of bacteria to detoxify methylglyoxal and reactive electrophiles.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—All strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37 °C in lysogenic broth (LB) or MOPS minimal medium (Teknova, Inc.) containing 0.2% glucose, 1.32 mM K₂HPO₄, and 10 μM thiamine. Unless specified otherwise, all chemicals were from Fisher or Sigma-Aldrich. N-Chlorotaurine and methylglyoxal were synthesized before each use (14, 15).
Microarray Analysis and Data Processing—E. coli MG1655 was grown in MOPS glucose medium at 37 °C with aeration to an A600 of 0.4–0.5. HOCl was added to a final concentration of 400 μM immediately before, 5 min after, and 10 min after HOCl addition, and total RNA was prepared using the Rneasy® Midi kit (Qiagen). cDNA synthesis, array hybridization to Affymetrix GeneChip E. coli genome 2.0 arrays, and imaging were performed at the Affymetrix and Microarray Core facility at the University of Michigan, Ann Arbor. Quality of raw images and expression values were analyzed using the expresso function and affy package of Bioconductor (16). Three biological replicates were conducted. The TM4 MultiExperiment Viewer (17) was used to identify patterns of gene expression and differentially expressed genes. A total of 5367 MG1655 probes were separated into 25 clusters with 100 iterations. The pattern of each cluster was evaluated based on log2-transformed ratios of treated versus untreated samples. Probes in each cluster have expression patterns similar to each other and dissimilar to those in other clusters.

Construction of E. coli Mutant Strains—DNA manipulations were conducted using standard methods (18). Primers are listed in Table 2. All constructs were confirmed by sequencing (GENEWIZ, Inc.). In-frame replacements of complete coding sequences with chloramphenicol resistance cassettes (19) were constructed for the following genes, using the indicated primers: nemR, [1] and [2]; nemA, [3] and [4]; gloA, [5] and [6]. The chloramphenicol resistance cassettes were resolved (19) to yield nonpolar in-frame deletions.

Sequence Analysis and Building of the NemR Model—Custom Python scripts using Biopython 1.57 were used to search for and sort NemR homologs from the National Center for Biotechnology Information databases (accessed 8/26/11). Alignments were performed using MUSCLE 3.8 (20) and visualized with WebLogo 3.1. NemR structure was modeled with SWISS-MODEL (21).
**NemR Is a Bleach-sensing Transcription Factor**

**TABLE 2**

| Primer | Sequence |
|--------|----------|
| [1]    | 5’-ATG AAC AAC CAT CAT GAT ACT TCC GAG TGT GCT GCT TC-3’ |
| [2]    | 5’-GCT CAT CAT AAG TAA AAC TGG CAC TCA TCT TTT TTT TTT ACC AGC TCA GCC GGC GCT TCC ATG GCC |
| [3]    | 5’-CTT CCT TCT ACT ACC GCG TGG CAC CAG GAC GGC GCG GCT CAG TCT CAA ATG |
| [4]    | 5’-ATT CAT AAA CAT TTT TCT TTT AAC TGG GCC GAT GAG GCT GAA GCA TAC |
| [5]    | 5’-ATG AAT ATC CTC CTT AG-3’ |
| [6]    | 5’-GTC TGC CCA GAT GAG CCA TCT GGT AG-3’ |
| [7]    | 5’-ATG CAT ATG AAC AAA AAC ACC GAA CAT G-3’ |
| [8]    | 5’-CTT aag ctt act acc gcg tgg cac ceg AAC GGC AGT CTC GCC AAT AAT-3’ |
| [9]    | 5’-TCC TCG TCA GAG AAC CAC TGA TG-3’ |
| [10]   | 5’-GAA AAT AAC GCT CTA ACC CCC TAA GAC GCA GAA CAT CAT GAT GCA-3’ |
| [11]   | 5’-GAC ATC GAT TTT TTA CGT GGT CAG-3’ |
| [12]   | 5’-TAA CAT TAC ACT ACC GCT CAG TTT GAG GAT AAA AAG ATG GTG-3’ |
| [13]   | 5’-TCA ACA TCT TGG CAT CAG-3’ |
| [14]   | 5’-GAA AAC TTA CCT GCT GAT CAT-3’ |
| [15]   | 5’-GTT TTA GTC TGG GCC AAC CAC TGA TA-3’ |
| [16]   | 5’-AGG TCG ATC AGT AGA AAA AAC AGC TG-3’ |
| [17]   | 5’-TAC TAC GTC CCA GAG AAC TCA TAG TCT CTA-3’ |
| [18]   | 5’-TAA ATG AAT ACC GCT CAA CAT GCA ATG GCT GCA-3’ |
| [19]   | 5’-GCT GCT GCA GCC TTT TTT ATC ACC AGA CGA CCG GGA GCC TTT ATG GCG-3’ |
| [20]   | 5’-GAA GCA GCT CCA GCC TAC ACC TAA ACG GCA GCA GTC GCA GAA CAT AT-3’ |
| [21]   | 5’-GAA ACC CCA TGG TTT ACC TTA CCA GCT GCT GCA-3’ |
| [22]   | 5’-GAC TGT TAC ACC CTA AAT ATC CTC CTT AG-3’ |
| [23]   | 5’-GAG GAT ATG AAT ATC CCA ATT GAG GAT AAA AAG ATG GTG-3’ |
| [24]   | 5’-AAG AAC TTA CCT GCT GAT CAT-3’ |
| [25]   | 5’-CAG GGC CAT ACT TCC ACC TCC TCT TCT TTT TCA ACC ACC GAA CAT G-3’ |
| [26]   | 5’-GTC TCG TCA GAG AAC CAC TGA TG-3’ |
| [27]   | 5’-GCA TGG TCC TCT ACT CCT CCA ACC TAG GCT GCA TTT TTT ATC ACC AGA CGA CCG GGA GCC TTT ATG GCG-3’ |
| [28]   | 5’-GAA GCA GCT CCA GCC TAC ACC TAA ACG GCA GCA GTC GCA GAA CAT AT-3’ |
| [29]   | 5’-GAC ATC GAT TTT TTA CGT GGT CAG-3’ |
| [30]   | 5’-ATC CAT CCA ACC TCA GCC GAA GCA GCA TGG TAT TAC AAG GAG AAA AAC TCA-3’ |
| [31]   | 5’-ATG AAT ATC CTC CTT AG-3’ |
| [32]   | 5’-ATG AAT ATC CTC CTT AG-3’ |
| [33]   | 5’-ATG AAC CAA CTT AAC GGC AGG CGT CGC AAT AAT-3’ |
| [34]   | 5’-ATG AAT ATC CTC CTT AG-3’ |
| [35]   | 5’-GAC ATC GAT TTT TTA CGT GGT CAG-3’ |
| [36]   | 5’-ATC CAT CCA ACC TCA GTC-3’ |
| [37]   | 5’-ATC CAT CCA ACC TCA GTC-3’ |

**Construction of nemR Plasmids**—The nemR gene, without the stop codon, was amplified from *E. coli* MG1655 with primers [7] and [8] and cloned into the Ndel and HindIII sites of plasmid pET-21b (Novagen) to generate the C-terminally His<sub>6</sub>-tagged NemR expression plasmid pNEMR1. The *nemR* gene and stop codon, plus 21 bp of 5’ sequence, were amplified from *E. coli* MG1655 with primers [9] and [10] and cloned into the EcoRI and HindIII sites of plasmid pBAD30 to generate plasmid pNEMR2. The QuikChange<sup>TM</sup> protocol (Stratagene) was used to mutate pNEMR1 with primers [11] and [12], [13] and [14], or [15] and [16]. This yielded plasmids pNEMR3, pNEMR7, and pNEMR8. The QuikChange protocol, modified by using only a single mutagenic primer and 35 cycles of amplification, was used to mutate pNEMR1 with primer [17], [18], or [19]. This yielded plasmids pNEMR12, pNEMR13, and pNEMR16. The QuikChange multisite-directed mutagenesis kit (Stratagene) was used to generate pNEMR2 with primers [13], [15], [17], and [19]. This yielded plasmid pNEMR32. The single-primer QuikChange procedure was used to mutate pNEMR2 and pNEMR32 with primer [11]. This yielded plasmids pNEMR43 and pNEMR44. The *nemR* alleles were amplified from pNEMR32 and pNEMR44 with primers [7] and [8] and cloned into the Ndel and HindIII sites of plasmid pET-21b<sup>+</sup> to yield plasmids pNEMR40 and pNEMR45.

**Construction of Chromosomal nemR Variants**—The nemR alleles from pNEMR2, pNEMR32, pNEMR43, and pNEMR44 were amplified using primers [20] and [21]. The cat<sup>+</sup> cassette was amplified from plasmid pKD3 (19) using primers [22] and [23]. 200 fmol of *nemR* PCR product and 200 fmol of cat<sup>+</sup> PCR product were mixed and used as template for amplification with primers [20] and [23]. The resulting nemR-cat<sup>+</sup> PCR products were gel-purified, transformed into strain MJG244, and selected for chloramphenicol resistance. The cat<sup>+</sup> cassettes in these strains were then resolved (19).

**Gene Expression Analysis by RT-PCR**—*E. coli* strains were grown at 37 °C with shaking in MOPS glucose medium at 37 °C to *A<sub>600</sub>* = 0.4–0.5, and oxidants were added as indicated. After defined time points, 0.5-ml samples were collected in liquid nitrogen. RNA was prepared using the RNeasy<sup>®</sup> Mini kit (Qiagen) and DNA-free<sup>TM</sup> kit (Ambion). SuperScript<sup>®</sup> III reverse transcriptase (Invitrogen) was used to generate cDNA, and RT-PCRs were set up with SYBR<sup>Green</sup> qRT-PCR mix (Invitrogen) and a Mastercycler<sup>®</sup> ep realplex2 real-time PCR system (Eppendorf). Expression ratios were calculated compared with expression of each gene in nonstressed MG1655 cultures by the ΔΔCt method (23) and normalized to expression of *rrsD*, encoding 16S rRNA, expression of which did not change under our conditions. Primers used for RT-PCR analysis were: *rrsD*, [24]...
and [25]; nemR, [26] and [27]; nemA, [28] and [29]; gloA, [30] and [31]; pck, [32] and [33]; yeaU, [34] and [35].

HOCl Survival Assays—*E. coli* MG1655 and isogenic mutant strains were grown at 37 °C with shaking in 10 ml of MOPS glucose medium to \( A_{600} = 0.4 – 0.6 \) and harvested by centrifugation. Cells were resuspended to \( A_{600} = 0.35 \) in 10 ml of MOPS glucose medium containing 2 mM HOCl in 125-ml baffled flasks and incubated at 37 °C with shaking (200 rpm). 0.5 ml of glucose medium containing 2 mM HOCl in 125-ml baffled flasks and incubated at 37 °C with shaking (200 rpm). 0.5 ml of cells was harvested by centrifugation immediately before and at defined time points after HOCl addition, then rinsed with MOPS medium containing 10 mM Na2S2O3, but no glucose, 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM KCl, 10 mM MgCl2, 0.1 mM EDTA, 10% glycerol. Samples were separated by nonreducing SDS-PAGE and visualized by Western blotting using an anti-His tag antibody (Abcam).

**RESULTS**

Identification of Bleach-responsive Regulators in *E. coli*—To identify regulators that might contribute to bacterial bleach defense, we examined gene expression in *E. coli* before and after a sublethal dose of HOCl (0.4 mM) using a transcriptional microarray, the full results of which are shown in *supplemental Table S1*. Among the genes significantly up-regulated by HOCl treatment were two electrophile detoxification genes (*nemA* and *gloA*) known to be under the control of NemR, a transcriptional repressor of the TetR family (Fig. 1A, inset). NemR, which was also found to be significantly up-regulated in response to HOCl treatment in our microarray analysis, has previously been reported to be sensitive to the electrophilic cysteine alkylating agent N-ethylmaleimide (NEM) (12). NEM is a synthetic reagent that irreversibly alkylates cysteine thiols. In contrast, HOCl is a physiological oxidant that causes the rapid and reversible oxidation of cysteine thiols to sulfenic acids and disulfide bonds (27). We therefore decided to pursue the study of NemR as a potentially bleach-responsive transcriptional regulator.

**NemR is a Bleach-sensing Transcription Factor**—Quantitative RT-PCR confirmed our microarray data and revealed approximately 70-fold up-regulation of *nemR* and 100-fold and 10-fold up-regulation of the downstream genes *nemA* and *gloA*, respectively, in response to sublethal HOCl treatment (Fig. 1A). Expression of both *nemA* and *gloA* was constitutively induced and almost completely nonresponsive to HOCl in a ∆*nemR* strain (Fig. 1A), indicating that bleach-mediated expression of both genes was almost entirely dependent on NemR. Although Umezawa et al. (12) identified weak NemR binding sites upstream of the pck and yeaU genes of *E. coli*, neither of these genes showed NemR-dependent up-regulation upon HOCl treatment (data not shown), suggesting that *nemA* and *gloA* are the primary regulatory targets of NemR.

To determine the *in vivo* oxidant specificity of the NemR response, we treated wild-type *E. coli* cells with various electrophiles and with reactive oxygen, nitrogen, and chlorine species, utilizing concentrations that induced transient growth arrest under our growth conditions without killing the cells (data not shown). These studies revealed that, in addition to responding
to NEM, NemR is sensitive to treatment with either HOCl or N-chlorotaurine, a prominent secondary oxidation product of HOCl in vivo (13). It also responded weakly to the disulfide-generating electrophile diamide (diazenedicarboxylic acid bis(N,N-dimethylamide)) (Fig. 1B) (28). In contrast, no significant NemR-mediated gene expression was observed in response to H$_2$O$_2$, the O$_2$ generator methyl viologen, the nitric oxide source diethylamine nitric oxide (DEANO), or, notably, methylglyoxal, the substrate of the NemR downstream target GloA (glyoxalase I) (29) (Fig. 1). These data demonstrate that induction of the nemR regulon is sensitive to HOCl and related RCS.

Phenotypic studies revealed that deletion of gloA and, to a lesser extent, nemA, increased the HOCl sensitivity of E. coli cells. In contrast, deletion of nemR had no significant effect on bleach survival (Fig. 2A), indicating that constitutive overexpression of nemA and gloA did not provide additional protection against HOCl. As expected, mutants lacking gloA were more sensitive to methylglyoxal treatment than wild-type E. coli (Fig. 2B) (29). A ΔnemR strain was considerably more resistant to methylglyoxal than wild type presumably due to the constitutive derepression of gloA. Similarly, pretreatment of E. coli with 0.4 mM HOCl protected against methylglyoxal stress (Fig. 2C), as would be expected due to NemR-dependent up-regulation of gloA. ΔnemA mutants showed no defect in survival of methylglyoxal stress, indicating that NemA is more important for HOCl stress tolerance than for resistance to exogenous methylglyoxal (Fig. 2, A and B). Consistent with these results, HPLC analysis of HOCl-treated E. coli revealed substantial accumulation of intracellular methylglyoxal after HOCl treatment (Fig. 2D). These results suggest that NemR is a bacterial repressor which is sensitive to HOCl and related RCS and whose gene products contribute to increased bleach resistance in E. coli by detoxifying reactive electrophiles produced during RCS stress.

Cysteine 106 Is Conserved among NemR Homologs—The simplest hypothesis explaining these results was that HOCl-mediated modification of NemR leads to the derepression of the nemR operon, causing the up-regulation of genes that increase bacterial resistance to these oxidants. To first investigate whether the NemR bleach sensing mechanism might involve oxidation of redox-sensitive metals (11), we conducted inductively coupled plasma mass spectrometry of the purified NemR protein. However, we were unable to detect any metals associated with NemR (data not shown), indicating that HOCl sensing in NemR is likely mediated by other means. Because some transcription factors, such as the peroxide-sensing OxyR, have been shown to rely on the reactivity of functionally or structurally important cysteine thiols to sense oxidants (9), we aligned NemR homologs from 166 bacterial genera (BLAST e-value < 0.00001) to identify potentially evolutionarily important cysteines. Of the six cysteines in E. coli NemR, only one, Cys-106, is highly conserved (Fig. 3A, E. coli numbering in red). Modeling of the E. coli NemR sequence onto the molecular structure of its Acinetobacter homolog (Protein Data Bank ID code 3KNW, 41% identical, 61% similar to E. coli NemR) (21)

FIGURE 1. A, E. coli MG1655 (wild-type) and the ΔnemR mutant grown to midlog phase in MOPS glucose medium and treated with 0.4 mM HOCl. Expression ratios of nemR (black ■), nemA (blue ●), or gloA (red □) were determined by RT-PCR (mean ± S.D. (error bars)). Inset, operon structure of the nem locus using the same color code. B, expression of nemR in E. coli MG1655 upon treatment with 0.4 mM HOCl, 0.2 mM N-chlorotaurine, 0.1 mM N-ethylmaleimide (NEM), 0.5 μM diamide, 2 μM H$_2$O$_2$, 0.4 mM methylviologen, 0.2 mM diethylamine nitric oxide (DEANO), or 0.2 mM methylglyoxal for 10 min relative to expression in the absence of any treatment (None) (mean ± S.D.).

FIGURE 2. A and B, E. coli MG1655 (wild-type) and mutant strains were incubated in MOPS glucose medium containing 2 mM HOCl for 150 min (A) or 150 μM methylglyoxal (MGO) for 120 min (B), then diluted and spot-titered on LB agar. C, E. coli MG1655 was incubated in MOPS glucose medium containing 0.4 mM HOCl for 10 min, followed by the addition of 150 μM methylglyoxal, then, after 150 min, diluted and spot-titered on LB agar. D, E. coli MG1655 was incubated in MOPS glucose medium containing 0.4 mM HOCl. Intracellular free methylglyoxal/cfu was measured by HPLC (mean ± S.D. (error bars)).
predicted that Cys-106 is surface-exposed and faces the predicted DNA binding site (Fig. 3B, green helices). Cys-21 is located directly in the DNA binding helix whereas Cys-116 and Cys-153 are located in the predicted dimerization interface of NemR. None of these other cysteines is, however, particularly well conserved among NemR homologs (Fig. 3A).

**Cys-106 Is Sufficient for NemR RCS-sensing Mechanism**—To investigate the role of these cysteines in the ability of NemR to respond to HOCl and related RCS, we constructed strains containing different cysteine mutant alleles of nemR at the native locus in the E. coli chromosome. As a read-out, we monitored their ability to respond to HOCl treatment with the overexpression of nemR using RT-PCR.

NemR<sup>C<sub>106S</sub></sup>, in which all six cysteines were mutated to serine, did not respond at all to HOCl treatment (Fig. 4, red trace), strongly indicating that HOCl sensing was cysteine-dependent. Repression of the nemR operon was maintained in these strains, albeit slightly relieved compared with to wild-type strains, indicating that this mutant protein largely maintains its DNA binding capacity despite the lack of all cysteines. A similar relief in basal repression was observed in strains expressing NemR<sup>G<sub>5C-S<sub>C<sub>106S</sub></sub></sup>, which lacked all but the absolutely conserved Cys-106. In contrast to the strain expressing the no-cysteine variant, however, this strain responded to HOCl in a wild-type-like manner (Fig. 4, compare black and green traces). These results suggested that the conserved Cys-106 is sufficient for mediating a wild-type-like HOCl response in NemR.

Notably, cells expressing NemR<sup>C<sub>106S</sub></sup>, a variant that lacks the conserved Cys-106 but contains all five other nonconserved cysteines, displayed a wild-type-like response to HOCl treatment (Fig. 4, blue trace). This finding further supports the importance of Cys-106 in the RCS-sensing mechanism of NemR.
cysteines, were also able to mount a HOCl response (Fig. 4, blue trace). However, gene expression rapidly declined in these bacteria, suggesting that Cys-106 might be crucial for maintaining a sustained HOCl response. This result was not completely unexpected given that some of the nonconserved cysteines are predicted to be in structurally or functionally critical locations of NemR and their oxidation might lead to derepression as well. Nevertheless, the fact that Cys-106 is absolutely conserved among members of the NemR family and is sufficient to confer the observed bleach response suggests that Cys-106 plays the central role in the NemR RCS-sensing mechanism.

NemR DNA Binding Activity Is RCS-sensitive in Vitro—To investigate the effects of RCS treatment on the DNA binding activity of NemR in vitro, we purified wild-type NemR and cysteine-mutated NemR variants and conducted in vitro gel shift assays using a DNA fragment containing the nemR promoter (Fig. 5A). We found that reduced wild-type NemR was treated with 1 mM DTT for 15 min to reduce reversible thiol modifications before incubation with DNA (A, lower right panel).
tion of NemR with a 1:1 molar ratio of N-chlorotaurine, used as an HOCl substitute in in vitro experiments because it causes many fewer nonspecific oxidation artifacts than HOCl (30), substantially decreased the NemR DNA binding affinity (Fig. 5A, top right). Incubation of NemR with a 1:1 molar ratio of H$_2$O$_2$ had much less effect on its DNA binding (Fig. 5A, bottom left), consistent with the failure of H$_2$O$_2$ to induce nemR expression in vivo (Fig. 1B). These results supported our in vivo studies and suggested that RCS oxidation of NemR causes its dissociation from DNA, therefore leading to the observed derepression of NemR target gene expression. Most importantly, incubation of oxidized inactive NemR with excess amounts of the thiol-specific reducing agent DTT fully restored DNA binding (Fig. 5A, bottom right), indicating that, unlike the irreversible inactivation of NemR by NEM alkylation (12), oxidative inactivation of NemR is a fully reversible process in vitro.

In vitro DNA binding analysis of the three NemR cysteine variants yielded results that were also fully consistent with our in vivo findings. The mutant variant NemR$_{5C-S(C106)}$, which contains only the conserved Cys-106, revealed a decrease in in vitro DNA binding affinity in response to N-chlorotaurine treatment that was very similar in relative extent to the decrease observed in N-chlorotaurine-treated wild-type NemR (Fig. 5B). The presence of all six cysteines except Cys-106 also resulted in in vitro RCS sensitivity (Fig. 5C), supporting our in vivo results suggesting that one or more of the remaining nonconserved cysteines of E. coli NemR are RCS-sensitive as well (Fig. 4). In contrast, the cysteine-free NemR$_{6C-S}$ protein remained bound to DNA independent of its pretreatment, indicating that absence of all six cysteines did not affect the NemR DNA binding but eliminated its ability to sense reactive chlorine species (Fig. 5D).

**Monitoring the in Vitro and in Vivo Thiol Oxidation Status of NemR**—To further examine the role that cysteine residues play in the RCS response of NemR, we examined the thiol oxidation status of our purified NemR wild-type and the three cysteine variants on nonreducing SDS-PAGE before and after treatment with N-chlorotaurine. In the absence of oxidants, all purified NemR variants migrated predominantly as monomeric proteins, although some disulfide-bonded species were already observed in untreated wild-type NemR (Fig. 5B). The single Cys-106 in NemR$_{5C-S(C106)}$, revealed that of the 80% of reduced molecules in the untreated NemR$_{5C-S(C106)}$ preparation, all but 8% were oxidized by N-chlorotaurine. Most notably, incubation of oxidized NemR$_{5C-S(C106)}$ with 5 mM DTT for 30 min reduced almost 60% of all molecules, excluding the presence of irreversible overoxidation products (such as sulfinic or sulfonic acids). The simplest hypothesis to explain this result was that, in the absence of other cysteines, Cys-106 might undergo reversible sulfenic acid formation, the immediate consequence of RCS-mediated chlorination of thiol groups (4). Both reduced thiols and sulfenic acids can be detected using the probe NBD-Cl, which forms adducts with absorption maxima of 420 nm for thiols and 350 nm for sulfenic acid (31). Indeed, Ellman’s assay, which we used to monitor the oxidation status of the single Cys-106 in NemR$_{5C-S(C106)}$, revealed that the 80% of reduced molecules in the untreated NemR$_{5C-S(C106)}$ preparation, 10% were oxidized by N-chlorotaurine. Most notably, incubation of oxidized NemR$_{5C-S(C106)}$ with 5 mM DTT for 30 min reduced almost 60% of all molecules, excluding the presence of irreversible overoxidation products (such as sulfinic or sulfonic acids). The simplest hypothesis to explain this result was that, in the absence of other cysteines, Cys-106 might undergo reversible sulfenic acid formation, the immediate consequence of RCS-mediated chlorination of thiol groups (4). Both reduced thiols and sulfenic acids can be detected using the probe NBD-Cl, which forms adducts with absorption maxima of 420 nm for thiols and 350 nm for sulfenic acid (31). We recorded the UV spectrum of N-chlorotaurine-oxidized NemR$_{5C-S(C106)}$ in the presence of NBD-Cl but were unable to detect any significant sulfenic acid adducts in our sample (data not shown). Depending on the microenvironment of the cysteine, however, the nucleophilicity of the sulfur in sulfenic acid may not be sufficient to react with the electrophilic NBD-Cl reagent. We therefore decided to conduct a chymotryptic digest of reduced and N-chlorotaurine-oxidized NemR$_{5C-S(C106)}$ and perform mass spectrometric analysis of the peptides. In contrast to the reduced NemR$_{5C-S(C106)}$ sample, for which 100% coverage was obtained, we were unable to identify
the Cys-106-containing peptide in the oxidized sample despite using an error-tolerant search with the latest version of Unimod (32). Future studies are clearly needed to identify the nature of the reversible thiol oxidation product in oxidized NemR.

In vivo thiol trapping experiments fully supported our in vitro findings and revealed the formation of intermolecular disulfide bonds in HOCl-treated cells expressing either wild-type NemR or the NemR variants lacking Cys-21, Cys-106, and Cys-149 (Fig. 6B). Variants lacking Cys-98 or Cys-153 formed substantially fewer higher molecular mass species, whereas the variant lacking Cys-116 formed none. These results indicate that Cys-116, located within the predicted dimer interface of NemR (Fig. 3B), plays the primary role in the observed intermolecular disulfide formation after HOCl stress in vivo. Neither the redox-active NemRSC-S(C106) variant nor the inactive NemRSC-S(C106) variant formed any disulfide bonded species in vivo. These results confirm that intermolecular disulfide bond formation is not required for a sustained NemR-mediated RCS response in E. coli and suggest that reversible oxidative modification of the absolutely conserved Cys-106 plays the central role in the sensing of and response to RCS.

**DISCUSSION**

Our study demonstrates that NemR is a transcriptional regulator that uses RCS-sensitive cysteine residues to rapidly induce expression of specific reductases involved in the detoxification of reactive electrophiles. Our data suggest a model in which NemR uses the reversible oxidation of its highly conserved Cys-106 as a trigger for conformational rearrangements that cause its dissociation from the DNA and subsequently the activation of nemA and gloA expression. Because in vitro exposure of wild-type NemR to an equimolar amount of N-chlorotaurine led not only to the oxidation of Cys-106, but also to the formation of a variety of intermolecularly disulfide-bonded oligomers (Fig. 6), it is likely that RCS-mediated chlorination of Cys-106 not only leads to oxidation of Cys-106 but also to an oxidative cascade, chlorinating and oxidizing other cysteine residues in NemR (4). In vivo analysis demonstrated that Cys-116, predicted to be near the dimer interface (Fig. 3B), is involved in the formation of intermolecular disulfide bonds (Fig. 6C) and may be responsible for the inactivation of NemR by RCS in the absence of Cys-106 (Figs. 4 and 5). However, the NemRSC-S(C106) mutant, which lacks Cys-116 (as well as all other cysteines apart from Cys-106), is able to respond to RCS in a wild-type manner both in vivo and in vitro, indicating that Cys-106, the only conserved cysteine in NemR, is sufficient for wild-type-like HOCl sensing in vivo. The additional nonconserved cysteine residues appear to be able to partially substitute for its absence. This result is reminiscent of the peroxide-sensing transcription factor OxyR, which uses reversible peroxide-mediated sulfenic acid formation at its absolutely conserved Cys-199 for sensing but is able to undergo disulfide bond formation with the less conserved Cys-208 (9). At this point, we do not know the nature of the reversible thiol modification that occurs at NemR Cys-106 and which leads to the loss in DNA binding affinity. Based on our preliminary mass spectrometric results, the modification appears to be distinct from the reported sulfenamide formation or S-bacillithiolation that occurs at Cys-15 of B. subtilis repressor OhrR. Like NemR, OhrR is inactivated by HOCl, leading to the derepression of its target gene, peroxiredoxin OhrA (33, 34). YjIE, another HOCl-sensing transcription factor in E. coli, contains no conserved cysteines (5), and the mechanism by which YjIE senses HOCl remains unknown. The role of the NemR nonconserved cysteines, and the properties of NemR that confer its specificity for certain electrophiles and RCS are currently under investigation.

Our studies revealed that NemA and particularly GloA are critical to bacterial bleach survival. NemA is a flavin-dependent reductase, which is active on a variety of electrophilic compounds in vitro, including NEM. However, its physiological substrate is so far unknown (35). The function of GloA is much better understood. GloA is broadly conserved among prokaryotic and eukaryotic species as the primary methylglyoxal-de toxifying enzyme (29). Methylglyoxal is a reactive ketohaldehyde capable of damaging proteins and nucleic acids and is formed by dephosphorylation of the glycolytic intermediate dihydroxyacetone phosphate (36). Numerous oxidative stress conditions have been associated with the accumulation of methylglyoxal and other reactive electrophiles in both bacteria and eukaryotes; however, the precise relationship between oxidative stress and methylglyoxal accumulation remains poorly understood (37). Our results demonstrate that increased methylglyoxal production is a direct consequence of HOCl stress and suggest that detoxification of reactive electrophiles is a critical component of bacterial RCS defense. The pathway(s) responsible for HOCl-induced methylglyoxal production are currently being investigated in our laboratory. The fact that NemR does not respond directly to methylglyoxal implies that E. coli uses NemR to mount a proactive response, anticipating an increase in methylglyoxal levels in response to RCS.

Previous work on NemR has shown that it responds to cysteine-modifying electrophiles, including NEM, showdowycin, and, more weakly, iodoacetamide (12). In combination with the results presented here, this suggests that NemR is an RCS- and electrophile-sensing regulator important for defense against a subset of stresses that result in toxic modification of cellular thiols. NemR homologs are broadly conserved among both Gram-negative and Gram-positive bacteria. The E. coli-like nemR operon structure is conserved among Enterobacteriaceae (38), suggesting that the NemR-controlled gloA and nemA expression response is conserved throughout this group. In other clades, genes encoding NemR homologs are commonly found in loci containing genes potentially involved in response to oxidative or electrophile stress, including glutathione S-transferases, oxidoreductases, and alcohol dehydrogenases. Supporting the idea that sensing of RCS may be a common function among NemR homologs, the Rhodococcus rhodochrous NemR homolog DhaR (31% identical, 51% similar to E. coli NemR) has been shown to regulate breakdown of a variety of chlorinated compounds, including 1-chlorobutane and 1,3-dichloropropene, although the detailed mechanism and specificity of DhaR have not been investigated (39).

In conclusion, our studies identified NemR as a broadly conserved bacterial transcription factor that relies on cysteine thiol oxidation to sense and respond to both RCS and reactive elec-
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trophies. In E. coli, NemR regulates expression of electrophile detoxification genes that play an important role in surviving bleach stress.

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REFERENCES

1. Rutala, W. A., and Weber, D. J. (1997) Uses of inorganic hypochlorite (bleach) in health-care facilities. Clin. Microbiol. Rev. 10, 597–610
2. Klebanoff, S. J. (2005) Myeloperoxidase: friend and foe. J. Leukoc. Biol. 77, 598–625
3. Bae, Y. S., Choi, M. K., and Lee, W. J. (2010) Dual oxidase in mucosal immunity and host-microbe homeostasis. Trends Immunol. 31, 278–287
4. Deborde, M., and von Gunten, U. (2008) Reactions of chlorine with inorganic and organic compounds during water treatment: kinetics and mechanisms. A critical review. Water Res. 42, 12–51
5. Gebendorfer, K. M., Dražic, A., Le, Y., Gündlach, J., Bepperling, A., Kastennmüller, A., Ganzinger, K. A., Braun, N., Franzmann, T. M., and Winter, J. (2012) Identification of a hypochlorite-specific transcription factor from Escherichia coli. J. Biol. Chem. 287, 6892–6903
6. Chi, B. K., Gronau, K., Máder, U., Hessling, B., Becher, D., and Antelmann, H. (2011) S-Bacillihiliolation protects against hypochlorite stress in Bacillus subtilis as revealed by transcriptomics and redox proteomics. Mol. Cell. Proteomics, 10.1074/mcp.M111.009506
7. Winterbourn, C. C., and Kettle, A. J. (2013) Redox reactions and microbial killing in the neutrophil phagosome. Antioxid. Redox Signal. 18, 642–660
8. Imlay, J. A. (2008) Cellular defenses against superoxide and hydrogen peroxide. Annu. Rev. Biochem. 77, 755–776
9. Vázquez-Torres, A. (2012) Redox active thiol sensors of oxidative and nitrosative stress. Antioxid. Redox Signal. 17, 1201–1214
10. Crack, J. C., Green, J., Hutchings, M. I., Thomson, A. J., and Le Brun, N. E. (2012) Bacterial iron-sulfur regulatory proteins as biological sensorswitches. Antioxid. Redox Signal. 17, 1215–1231
11. Spira, S., and D’Autréaux, B. (2012) Non-heme iron sensors of reactive oxygen and nitrogen species. Antioxid. Redox Signal. 17, 1264–1276
12. Umezawa, Y., Shimada, T., Kori, A., Yamada, K., and Ishihama, A. (2008) The uncharacterized transcription factor YdhM is the regulator of the nemA gene, encoding N-ethylmaleimide reductase. J. Bacteriol. 190, 5890–5897
13. Nagl, M., Hess, M. W., Pfäffer, K., Hengster, P., and Gottardi, W. (2000) Bactericidal activity of micromolar N-chlorotaurine: evidence for its antimicrobial function in the human defense system. Antimicrob. Agents Chemother. 44, 2507–2513
14. Kellum, M. W., Oray, B., and Norton, S. J. (1978) A convenient quantitative synthesis of methyglyoxal for glyoxalase I assays. Anal. Biochem. 85, 586–590
15. Peskin, A. V., and Winterbourn, C. C. (2001) Kinetics of the reactions of chlorine with organic and organic compounds during water treatment: kinetics and mechanisms. A critical review. Water Res. 42, 12–51
16. Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A. I., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, Y. J., and Zhang, J. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80
17. Saeed, A. I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Brasaitis, J., Klapa, M., Currier, T., Thigaranjan, M., Sturm, A., Snuffin, M., Rezants, A., Popov, D., Rytskov, A., Kostokuvich, E., Borisovski, I., Liu, Z., Vinsavich, A., Trush, V., and Quackenbush, J. (2003) TM4: a free, open-source system for microarray data management and analysis. BioTechniques 34, 374–378
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY