Response of *Pseudomonas aeruginosa* to the innate immune system-derived oxidants hypochlorous acid and hypothiocyanous acid

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Running title: Response of *P. aeruginosa* to HOCl and HOSCN

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ABSTRACT

*Pseudomonas aeruginosa* is a significant nosocomial pathogen and associated with lung infections in cystic fibrosis (CF). Once established, *P. aeruginosa* infections persist and are rarely eradicated despite host immune cells producing antimicrobial oxidants, including hypochlorous acid (HOCl) and hypothiocyanous acid (HOSCN). There is limited knowledge as to how *P. aeruginosa* senses, responds to, and protects itself against HOCl and HOSCN, and the contribution of such responses to its success as a CF pathogen. To investigate the *P. aeruginosa* response to these oxidants we screened 707 transposon mutants, with mutations in regulatory genes, for altered growth following HOCl exposure. We identified regulators of antibiotic resistance, methionine biosynthesis and catabolite repression, and PA14_07340, the homologue of the *Escherichia coli* HOCl-sensor RclR (30% identical), that are required for protection against HOCl. We have shown that RclR (PA14_07340) protects specifically against HOCl and HOSCN stress, and responds to both oxidants by upregulating expression of a putative peroxiredoxin, *rclX* (PA14_07355). Transcriptional analysis revealed that while there was specificity in the response to HOCl (231 genes upregulated) and HOSCN (105 genes upregulated) there was considerable overlap, with 74 genes upregulated by both oxidants. These included genes encoding the type III secretion system, sulphur and taurine transport, and the MexEF-OprN efflux pump. RclR coordinates part of the response to both oxidants, including upregulation of pyocyanin biosynthesis genes, and in the presence of HOSCN, downregulation of chaperone genes. These data indicate that the *P. aeruginosa* response to HOCl and HOSCN is multifaceted, with RclR playing an essential role.

IMPORTANCE
The bacterial pathogen *Pseudomonas aeruginosa* causes devastating infections in immunocompromised hosts, including chronic lung infections in cystic fibrosis patients. To combat infection the host’s immune system produces the antimicrobial oxidants hypochlorous acid (HOCl) and hypoiodite (HOSCN). Little is known about how *P. aeruginosa* responds to and survives attack from these oxidants. To address this, we carried out two approaches: a mutant screen and transcriptional study. We identified the *P. aeruginosa* transcriptional regulator, RclIR, which responds specifically to HOCl and HOSCN stress, and is essential for protection against both oxidants. We uncovered a link between the *P. aeruginosa* transcriptional response to these oxidants and physiological processes associated with pathogenicity, including antibiotic resistance and the type III secretion system.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a leading cause of nosocomial infections that are life threatening and difficult to treat (1). As an ESKAPE pathogen it is one of the bacteria that poses the greatest public health threat (2). Furthermore, *P. aeruginosa* is the major pathogen associated with chronic lung infections in patients suffering from cystic fibrosis (CF) (3, 4). This genetic disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator, which results in abnormal ion and water transport across epithelial membranes, and leads to dehydrated airways and the establishment of respiratory infections (5). The immune response to infection is characterised by persistent neutrophil-dominated inflammation that is ineffective at clearing infection and results in progressive lung tissue damage (3, 4).

Host cells defend themselves against invading pathogens by the production of oxidants during the innate immune response, including the hypohalous acids: hypochlorous acid (HOCl),
the active ingredient in bleach, and hypothiocyanous acid (HOSCN) (6). HOCl is a potent oxidant produced by neutrophils (7). The neutrophil oxidative burst that follows phagocytosis involves the reduction of O$_2$ to superoxide (O$_2^-$) by the enzyme NADPH oxidase and the dismutation of O$_2^-$ to hydrogen peroxide (H$_2$O$_2$) (7). The haem enzyme myeloperoxidase (MPO) catalyses the formation of HOCl from the reaction of H$_2$O$_2$ with chloride (Cl$^-$) (7). MPO can also mediate H$_2$O$_2$-oxidation of other halides including the pseudohalide thiocyanate (SCN$^-$) to HOSCN. However, its primary physiological substrate in neutrophils is proposed to be HOCl as the plasma concentration of Cl$^-$ (100-140 mM) is much greater than SCN$^-$ (20-100 µM) (6, 8). The physiological concentration of HOCl is unknown, due to the instability of this compound (6). At the epithelial cell surface of the lungs HOSCN is produced through the concerted action of the dual oxidase (DUOX) and lactoperoxidase (LPO) (9). The epithelial cell DUOX releases H$_2$O$_2$ into the airway surface liquid where LPO catalyses the reaction of H$_2$O$_2$ with SCN$^-$ to form HOSCN (9). HOCl can target proteins, lipids, DNA and RNA; the most reactive targets of HOCl are thiol (S-H) groups of cysteine and methionine (7). Oxidation of cysteine by HOCl results in the formation of sulphenic acid, which can react with other cysteine thiols to form reversible disulphide bonds, and oxidation of methionine by HOCl results in formation of methionine sulphoxide (6). Similarly to HOCl, HOSCN reacts fastest with thiol groups of cysteine forming sulphenic acids or disulphide bonds (6). However, HOCl is a stronger oxidant than HOSCN, with rate constants for cysteine thiol groups of $3.2 \times 10^7$ M$^{-1}$S$^{-1}$ and $7.8 \times 10^4$ M$^{-1}$S$^{-1}$, respectively (6).

Despite HOCl and HOSCN having greater antibacterial activities than H$_2$O$_2$, studies of the bacterial mechanisms used to protect against oxidative stress have, until recent years, focussed on the less potent oxidants, H$_2$O$_2$ and O$_2^-$ (6, 10). However, over the last 7 years
emerging studies into the bacterial response mechanisms to HOCl have identified thiol-based redox-sensing transcriptional regulators (11, 12). They include the *Bacillus subtilis* transcriptional factors OhrR, HypR and PerR and the *E. coli* transcriptional factors NemR, HypT and RclR (13-17). HypT and RclR are the first described bacterial transcriptional regulators that are specific to sensing HOCl (16, 17); the others respond to a variety of stresses including reactive electrophile species and organic hydroperoxides (11, 14, 15, 18-24). Identification of these HOCl-sensing regulators revealed that bacteria have specific mechanisms for responding to and protecting against HOCl stress, and do not rely solely on mechanisms used against other oxidants. Virtually nothing is known about bacterial responses to HOSCN, apart from a study from Groitl, et al. that investigated the transcriptional response of *P. aeruginosa* PA14 to HOSCN, as well as to HOCl and another hypohalous acid, hypobromous acid (HOBr) (25). A key finding of this study was that all three oxidants caused protein aggregation and *P. aeruginosa* responded by increasing polyphosphate levels, which protected against protein aggregation and aided survival (25).

Yet, there is still very little known about how the clinically relevant pathogen *P. aeruginosa* senses, responds to, and protects against HOCl or HOSCN. Due to *P. aeruginosa* encountering these innate immune cell-derived oxidants in a variety of infection environments, including the neutrophilic environment of the CF lung, it is important to increase our knowledge of these specific oxidative stress responses.

Here, we have used a combination of mutant screening and transcriptomics to identify genes and systems used by *P. aeruginosa* to survive HOCl and HOSCN challenge. Our major finding is the identification of the *P. aeruginosa* RclR system that is specifically required for protection against both HOCl and HOSCN stress, in addition to discovering regulators with
known roles in antibiotic resistance, methionine and carbon metabolism as having protective roles against HOCl.

RESULTS

Screening transposon mutants of *P. aeruginosa* regulatory genes for altered susceptibility to HOCl

We reasoned that targeting regulatory systems would be an effective way to uncover processes used by *P. aeruginosa* to protect against HOCl stress. To address this, we used mutants from the PA14 non-redundant transposon insertion mutant library (26), which contains 5,850 mutants from 4,596 genes (out of the 5,977 annotated genes) in the PA14 chromosome. To compile a subset of available mutants from this library, in genes classed as having confirmed or predicted regulatory roles, we used gene ontology information from the *Pseudomonas* genome database (27). A search for mutants in transcriptional regulator, two-component system, catalase, ser/thr kinase, sigma factor and other regulatory genes resulted in a subset of 707 mutants from 572 genes (Table S1). These 707 mutants were screened for altered susceptibility compared to wild-type (WT) when grown in the presence of 4.4 mM HOCl over 24 hours. The most sensitive strains were identified as those that consistently failed to grow during the time of the assay or had an increased lag >3 hours compared to WT. Resistant strains were identified as those that consistently had a decreased lag >3 hours compared to WT. Fig. 1 shows an example of a 96-well screening plate with HOCl-sensitive and HOCl-resistant mutants labelled. Identified HOCl-sensitive and HOCl-resistant mutants were rescreened and those consistently displaying altered HOCl-susceptibility are shown in Fig. S1. Statistical analysis on these data revealed 16 mutants...
that were significantly HOCl-sensitive and 13 mutants that were significantly HOCl-resistant compared to WT at set time-points (Tables 1 and 2).

**Links between antimicrobial resistance and HOCl susceptibility; the MexEF-OprN multidrug efflux pump protects \textit{P. aeruginosa} from HOCl**

A mutant in \textit{mexT} was unable to grow in the presence of HOCl (Fig. 2A). MexT is a LysR-type regulator that positively regulates expression of its adjacent operon \textit{mexEF-ooprN}, which encodes a multidrug efflux pump \cite{28, 29}. MexT regulates a number of genes \cite{30, 31}, in addition to the \textit{mexEF-ooprN} operon, and so to determine whether loss of the efflux pump is associated with HOCl-sensitivity, transposon mutants of all three efflux pump genes were tested for altered HOCl susceptibility; they displayed increased sensitivity in the order of \textit{mexE} > \textit{mexF} > \textit{oprN} (Fig. 2A). For all three mutant strains, the transposon is inserted within the middle of the gene. This suggests that the MexEF-OprN efflux pump provides \textit{P. aeruginosa} with protection against HOCl. MexT positively regulates 12 other genes \cite{30} in addition to \textit{mexEF-ooprN} and \textit{pyeR} (mentioned below); mutants in 8 of these were available for screening, and 4 displayed HOCl-sensitivity (PA14\_41990, PA14\_39060, PA14\_27755 and PA14\_27770), albeit none were as sensitive as \textit{mexE} (Table S2).

Another multidrug efflux pump regulator, NfxB, was required for protection against HOCl, as an \textit{nfxB} mutant had increased HOCl-sensitivity (Table 1 and Fig. 2B). NfxB is a negative regulator of the \textit{mexCD-ooprJ} multidrug efflux operon \cite{32} and transposon mutants in these genes were tested; only \textit{oprJ} had altered susceptibility, displaying HOCl-sensitivity (Fig. 2B).
MexT regulates the *pyeRM*-xenB operon, in which *pyeR* encodes an ArsR-family repressor, *pyeM* a major facilitator superfamily (MFS) transporter and *xenB* an uncharacterized xenobiotic reductase (33). Transposon mutants in *pyeR* showed variable susceptibility to HOCl (Fig. S1); therefore, we constructed a whole operon in-frame deletion mutant, Δ*pyeRM*-xenB, which displayed HOCl-sensitivity (Fig. 2C). This suggests a role for this MexT-regulated operon in protecting *P. aeruginosa* against HOCl.

Metabolic regulators are involved in protection against HOCl

A mutant in *metR* that encodes a regulator homologous (40% identity) to *E. coli* MetR, a regulator of methionine biosynthesis genes (34), was HOCl-sensitive (Fig. 3A). A mutant in PA14_07110 (*sahR*) was also HOCl-sensitive (Fig. 3A). This gene encodes an ortholog of SahR from *Desulfovibrio spp*, which is a transcriptional regulator of the S-adenosylmethionine cycle genes that are involved in methionine recycling (35).

The CbrA/B two component system is involved in controlling carbon and nitrogen metabolism, and carbon catabolite repression (36-38). CbrA/B acts through activation of the small RNA CrcZ, which sequesters the RNA chaperone Hfq and the catabolite repression control protein Crc from mRNA target genes, relieving catabolite repression (38-40) (Fig. 3B). Mutants of *cbrA/B* were HOCl-resistant (Fig. 3C) and subsequently the *crc* transposon mutant was tested and found to be HOCl-sensitive (Fig. 3D). These phenotypes correlate with the opposing regulatory roles of CbrA/B and Crc.
Regulators with putative roles in the oxidative stress response are required for protection against HOCl

A mutant in the \textit{algH} gene, which encodes a hypothetical protein that has been crystallised and predicted to be a potential redox-sensor, was HOCl-sensitive (Fig. 4A) \cite{41}. \textit{algH} is part of a 4-gene operon that includes \textit{gshB}, which encodes a glutathione synthetase; a \textit{gshB} mutant was tested and displayed HOCl-sensitivity (Fig. 4A).

A mutant in \textit{oxyR} encoding an H$_2$O$_2$-sensing transcriptional regulator \cite{42} was non-viable, however a mutant was available for its adjacent gene, a putative DNA repair enzyme, \textit{recG} \cite{42} and was HOCl-sensitive (Fig. 4B). The sensitivity of the \textit{recG} mutant to HOCl was not due to disruption of \textit{oxyR}, as RT-PCR confirmed the \textit{oxyR} transcript was present in the \textit{recG} mutant (data not shown). We constructed an in-frame deletion of \textit{oxyR}, which displayed HOCl-sensitivity (Fig. 4B). This suggests that the \textit{oxyR-recG} operon is involved in protection of HOCl, as well as H$_2$O$_2$ \cite{42}.

\textit{P. aeruginosa} mutants of homologues of other transcriptional regulators that respond to HOCl in bacteria, NemR (PA14\_36300), HypT (PA14\_71640) and OhrR (PA14\_27230), did not display consistent HOCl-sensitive or resistant phenotypes in the screen (Fig. S2). However, a mutant in PA14\_07340, the \textit{P. aeruginosa} homologue of the \textit{E. coli} reactive-chlorine specific transcriptional regulator, RclR \cite{17} was identified as HOCl-sensitive (Fig. 4C). The \textit{rclR} mutant was one of three strains from the screen that showed consistent, reproducible sensitivity to HOCl, and displayed significant altered growth in the presence of the oxidant when rescreened (Fig. 4C), but did not show significant altered growth at the time-points analysed in the initial screen (Table 1). \textit{P. aeruginosa rclR} (PA14\_07340) is adjacent to a single-gene operon.
PA14_07355 that encodes a putative peroxiredoxin enzyme of the alkylhydroperoxidase (AhpD) family (Fig. 4C). A mutant in PA14_07355 was tested and displayed HOCl-sensitivity (Fig. 4C).

**P. aeruginosa** RclR is specifically required for protection against HOCl and HOSCN

To confirm the role of *P. aeruginosa* RclR in protection against HOCl, in-frame deletion mutants of rclR and the putative peroxiredoxin gene, which we named rclX, were constructed. The susceptibility of the mutants to HOCl was retested; ΔrclR and ΔrclX grew similarly to WT in the absence of oxidant (Fig. 5A), but neither mutant grew in the presence of HOCl over the time-course of the experiment (Fig. 5B). Complementation of ΔrclR and ΔrclX with rclR-pUCP18 and rclX-pUCP18, respectively, restored growth in the presence of HOCl to that of WT (Fig. 5B). To test the specificity of RclR, mutants were exposed to other reactive oxygen species (H₂O₂, the O₂ generator methyl viologen and tert-butyl hydroperoxide (TBH)), reactive electrophilic species (N-ethylmaleimide (NEM), methylglyoxal and diamide) and the nitric oxide generator diethylamine NONOate (DEANO). These reactive species did not alter the growth of ΔrclR compared to WT, indicating that *P. aeruginosa* RclR is not required for protection against these compounds (Fig. S3). Due to HOSCN being another physiologically relevant, thiol-reactive oxidant produced by the immune system (9), we were interested to determine whether ΔrclR and ΔrclX had altered sensitivity to this oxidant. Growth of ΔrclR and ΔrclX in the presence of 0.8 mM HOSCN revealed ΔrclR was sensitive to HOSCN, whereas ΔrclX was not at this concentration (Fig. 5C). Complementation of ΔrclR with rclR-pUCP18 restored growth in the presence of HOSCN (Fig. 5C). These data were supported by viability assays, which showed HOCl inhibited growth of ΔrclR and ΔrclX (Fig. 5D), and HOSCN was bactericidal towards ΔrclR (Fig. 5E). When the HOSCN concentration was increased to 1 mM, ΔrclX, as well as
ΔrclR, displayed sensitivity to this oxidant (Fig. S4). Therefore, both RclR and RclX are required for protection against HOCl and HOSCN.

The sensitivity assays thus far were performed on planktonic cells in Luria-Bertani (LB) medium; to determine whether the findings were consistent when grown under conditions relevant to the CF lung environment, sensitivity assays for ΔrclR and ΔrclX mutants were repeated in (Artificial Sputum Medium) ASM, which mimics the CF sputum (43, 44). Both ΔrclR and ΔrclX displayed HOCl and HOSCN sensitivity when grown in ASM (Fig. S5), confirming the findings of the LB assays. Additionally, during chronic CF lung infections *P. aeruginosa* persists in biofilms. To determine whether *P. aeruginosa* mutants display similar HOCl and HOSCN susceptibility when forming biofilms, ΔrclR and ΔrclX mutants were grown in a suspended ASM biofilm assay in the absence or presence of HOCl and HOSCN (adapted from (44)). The WT, ΔrclR and ΔrclX strains formed biofilms in ASM in the absence of the oxidants (Fig. S6). However, in the presence of 3.5 mM HOCl and 0.53 mM HOSCN, both ΔrclR and ΔrclX were unable to grow and appeared sensitive to these oxidants, compared to WT that was able to form biofilms at these concentrations (Fig. S6). These data confirm the relevance of the impact of HOCl and HOSCN on *P. aeruginosa* in a more realistic CF environment.

**RclR regulates induction of rclX expression in response to HOCl and HOSCN stress**

To determine whether RclR regulates expression of itself and/or rclX under HOCl and HOSCN stress, transcriptional rclR-lacZ and rclX-lacZ fusions were constructed and introduced into the WT and ΔrclR strains for *in vivo* β-galactosidase assays. The activity of rclX-lacZ increased following HOCl and HOSCN exposure in the WT strain, but not in the ΔrclR strain, suggesting expression of rclX increases following HOCl and HOSCN stress dependent on RclR.
regulation (Fig. 6A). No difference in the activity of rclR-lacZ was observed between WT and ΔrclR strains in the absence or presence of HOCl and HOSCN, suggesting RclR does not autoregulate its own expression and is constitutively expressed (Fig. 6A). We next examined if the activation of rclX in response to these oxidants occurred in clinical CF isolates of P. aeruginosa. The activity of rclX-lacZ in three CF isolates was measured in the absence or presence of HOCl and HOSCN. The rclX gene was significantly induced in response to both oxidants in all three clinical isolates compared to untreated controls (Fig. 6B). This confirmed that rclX induction appears an important adaptive response to these oxidants across P. aeruginosa laboratory and clinical strains.

To investigate the possible function of RclX, we performed homology modelling that fitted the RclX sequence with 32.1% sequence identity to the AhpD-like protein, Lpg0406, from Legionella pneumophila, which belongs to the carboxymuconolactone decarboxylase family (45) (Fig. S7). The predicted structure was a homohexamer (Fig. S7A), formed from monomers with an AhpD-like fold containing six α-helices and a conserved catalytic CXXC motif (Fig. S7B).

RclX was sequence aligned with Lpg0406, PA0269 another AhpD-like protein from P. aeruginosa (46), and MtAhpD from Mycobacterium tuberculosis (47-49) (Fig. S7C). Four out of the five catalytic residues of MtAhpD responsible for peroxidase activity (47, 48) were conserved in RclX (Fig. S7C), suggesting a possible role for RclX in oxidant detoxification.

Transcriptional changes in PA14 WT and ΔrclR in response to HOCl and HOSCN exposure

The response of P. aeruginosa to HOCl and HOSCN was further characterised by measuring changes in gene expression in the WT and ΔrclR strains. PA14 WT and ΔrclR
exponential phase cultures were exposed to 2.2 mM HOCl or 0.8 mM HOSCN and gene expression analysed by RNAseq. Genes with a log2 fold change of >1.5 were considered upregulated and genes with a log2 fold change of < -1.5 were considered downregulated (Fig. 7). All transcriptomic data are presented in Data set S1 and are accessible through the Gene Expression Omnibus (GEO) database under accession number GSE124385. For identification of genes whose expression is altered in the presence of HOCl or HOSCN, expression was compared in WT treated strains versus WT untreated strains. HOCl upregulated 231 genes and downregulated 20 genes in the WT strain (Fig. 7A). HOSCN upregulated 105 genes and downregulated 16 genes in the WT strain (Fig. 7B). The importance of rclX in the P. aeruginosa response to HOCl and HOSCN was confirmed as it was strongly upregulated >6 and >8 log2 fold change, respectively, and this was consistent with the gene fusion expression data (Fig 6 and Fig. 7A+B). These data have been validated by qRT-PCR for a subset of the most highly upregulated and downregulated genes (including rclX), whose pattern of expression was confirmed (5 upregulated and 1 downregulated) (Fig. S8). For identification of genes whose expression is dependent on RclR, expression was compared in WT treated strains versus ΔrclR treated strains. Therefore, in Fig. 7C+D, genes displaying increased expression indicate genes positively regulated by RclR and genes displaying decreased expression indicate genes negatively regulated by RclR. In the presence of HOCl, RclR upregulated 42 genes and downregulated 7 genes (Fig. 7C), and in the presence of HOSCN, RclR upregulated 132 genes and downregulated 213 genes (Fig. 7D). Regulation of rclX by RclR was confirmed as it was the gene most strongly upregulated by RclR in the presence of HOCl and HOSCN (Fig. 7C+D). Expression of rclR was not altered following exposure to HOCl or HOSCN, agreeing with our previous gene fusion data (Data set S1 and Fig. 6A). Furthermore, the genes of homologues of
other HOCl-responsive transcriptional regulators from *E. coli* and *B. subtilis*; NemR (PA14_36300), HypT (PA14_71640) and OhrR (PA14_27230) did not have altered expression in response to HOCl or HOSCN (Data set S1).

The upregulated and downregulated genes were examined for overlap between the response to both oxidants and were categorised into functional groups based on assigned gene ontologies in the *Pseudomonas* genome database (27) (Fig. S9). Of the 105 genes upregulated in response to HOSCN, 74 (70%) were also upregulated in response to HOCl, indicating similarities between the bacterial responses to these two oxidants (Fig. S9A). The functional groups that displayed the largest percentage of genes upregulated in response to HOCl and HOSCN were non-coding RNA, protein secretion/export apparatus, and antibiotic resistance and susceptibility (Fig. S9B). Of the 132 RclR-regulated genes whose expression was upregulated in the presence of HOSCN only 12 (9%) of those were also upregulated in the presence of HOCl (Fig. S9C), and of the 213 RclR-regulated genes downregulated following HOSCN exposure only 2 (1%) were downregulated after HOCl exposure (Fig. S9D). For RclR-regulated genes the functional group with the highest percentage of genes upregulated following HOCl and HOSCN exposure was secreted factors (Fig. S9E). RclR downregulated a large number of genes in the presence of HOSCN, particularly those in the chaperone and heat shock proteins functional group (Fig. S9F). The observed regulatory effects might be a direct consequence of loss of RclR-mediated regulation or a result of secondary pathways impacted by *rclR* deletion.

tRNA, T3SS, sulphur and taurine metabolism, and MexT-regulated efflux pump genes show increased expression following exposure to HOCl and HOSCN.
Expression of functionally categorised genes was displayed in heat maps to demonstrate differences in gene expression in response to HOCl (first column) or HOSCN (second column) and in which upregulated genes are displayed in blue and downregulated in yellow (Fig. 8 and Fig. 9). Genes that are positively or negatively regulated by RclR are indicated by blue or yellow, respectively, in the presence of HOCl (third column) or HOSCN (fourth column) (Fig. 8 and Fig. 9).

The genes of non-coding RNAs were upregulated in response to HOCl and HOSCN; HOCl increased expression of tRNAs (Fig. 8A), whereas HOSCN upregulated 5s and 16s rRNAs (Data set S1). An rRNA depletion step had been carried out prior to RNAseq using the highly efficient Ribozero kit (50) and less than 2% rRNA was recovered, therefore the physiological importance of this induction is unclear. All of the genes that are involved in biogenesis and regulation of the type III secretion system (T3SS) (51) were induced in WT in response to both HOCl and HOSCN, while RclR appeared to downregulate a number of the T3SS genes in the presence of HOSCN (Fig. 8B and Data set S1). Around 40% of the genes in the transport of small molecules category that were upregulated in the WT response to HOCl and HOSCN were involved in sulphur or taurine metabolism (Fig. S9B, Fig. 8C and Data set S1). The mexEF-oprN operon, which encodes the efflux pump we found plays a role in protection against HOCl (Fig. 2A), was upregulated in response to HOCl and HOSCN exposure (Fig. 8D, and Data set S1). The mexT regulator did not have altered gene expression (Fig. 8D and Data set S1). Surprisingly, these data indicated that RclR downregulates expression of the mexEF-oprN operon in the presence of HOCl and HOSCN (Fig. 8D and Data set S1). The MexT-regulated operon, pyeRM-xenB, which is also involved in protection against HOCl (Fig. 2C), was upregulated in response.
RclR regulates expression of pyocyanin biosynthesis genes in the presence of HOCl and HOSCN, and denitrification and chaperone genes in the presence of HOSCN

Heat maps of functionally categorised genes with altered expression in the ∆rclR deletion mutant included phz, denitrification and chaperone genes. The phz genes encode for biosynthesis of the virulence factor pyocyanin (52). The overall WT response to HOCl and HOSCN had no and little effect, respectively, on phz expression (Fig. 9A and Data set S1). However, the ∆rclR mutant displayed lower phZ expression levels after HOCl and HOSCN exposure compared to WT, thus when values of WT treated were divided by ∆rclR treated the results indicate that RclR positively regulates phz gene expression in the presence of both oxidants (Fig. 9A and Data set S1). In the absence of these oxidants, there was no difference between pyocyanin production in WT and ∆rclR strains (data not shown). Similarly, the virulence factors elastase (lasB) and rhamnolipids (rhlA and rhlB) and an aminopeptidase (PA14_26020) did not display altered expression in response to HOCl or HOSCN in WT, but were upregulated by RclR in the presence of both oxidants (Fig. 9A and Data set S1). In the WT strain HOCl and HOSCN had little effect on expression of genes required for anaerobic dissimilatory denitrification (Fig. 9B and Data set S1). However, in the presence of HOSCN, but not HOCl, RclR positively regulated expression of 6 operons encoding the denitrifying enzymes: nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS) (Fig. 9B and Data set S1). HOCl and HOSCN had little effect on expression of chaperone and heat shock genes in the WT strain (Fig.
DISCUSSION

A major mechanism employed by the innate immune response to attack infecting bacteria, such as during lung infections, is through the production and release of the antimicrobial oxidants HOCl and HOSCN by neutrophils and the airway epithelium, respectively (7, 9).

In this study, we investigated how P. aeruginosa adapts to and protects itself against HOCl and HOSCN. This is of direct relevance to CF infections due to evidence of a compromised immune response in CF patients, including impaired HOCl and HOSCN formation (53-57), and evidence for HOSCN production being protective of lung function (58).

Our mutant screen identified regulators with known or putative roles in antibiotic resistance, methionine biosynthesis, catabolite repression and the antioxidant response that are required for protecting P. aeruginosa against HOCl (Fig. 2-4). We revealed that the RclR transcriptional regulator, the P. aeruginosa homologue of the E. coli RclR HOCl-specific sensor (17), is required specifically for protection of P. aeruginosa against HOCl and HOSCN and responds to the presence of these oxidants through activating expression of its adjacent gene, rclX (Fig. 5-7).
E. coli RclR (RclREc) also induces expression of its adjacent operon, rclABC, in the presence of HOCl (17). However, unlike rclREc (17), we found rclRPa is constitutively expressed, not upregulated by HOCl (nor HOSCN), and does not regulate its own expression (Fig. 6A). The rclX gene regulated by RclRPa encodes a putative peroxiredoxin and is one of the most upregulated genes in response to HOCl and HOSCN exposure and is required for protection against both oxidants (Fig. 5-7 and Fig. S4). RclX is not homologous to any of the genes in the RclREc-regulated rclABC operon (17), indicating differing approaches to protection against HOCl between these gram-negative bacteria. Homology modelling and multiple sequence alignment support RclX being an AhpD-like protein, with 4 conserved catalytic residues including the CXXC motif (Fig. S7), which is required for peroxidase activity in the functionally characterised AhpD from M. tuberculosis (47, 48). Therefore, it is probable RclX acts as a detoxification enzyme in the protective response to HOCl and HOSCN. The sensitivity of ΔrclR and ΔrclX mutants to HOCl and HOSCN when grown in ASM in both planktonic and biofilm cultures (Fig. S5-6), together with HOCl and HOSCN dependent expression of rclX in clinical CF isolates of P. aeruginosa (Fig. 6B), indicate that the RclR-mediated response is important in clinical strains and may play a role during infection.

There was considerable overlap in the transcriptional response of P. aeruginosa to HOCl and HOSCN (Fig. S9A+B). RclR regulated expression of a larger number of genes in the presence of HOSCN than HOCl (Fig. S9C+D). HOSCN caused RclR-dependent upregulation of pyocyanin biosynthesis genes and denitrification genes, and downregulation of chaperone and heat shock genes (Fig. 9). Three other transcriptional regulators that are HOCl-responsive in other bacteria: NemR (PA14_36300), HypT (PA14_71640) and OhrR (PA14_27230) (13, 15, 16), did not have altered expression in response to HOCl or HOSCN (Data set S1). In a previous
transcriptome study, Groitl et al., reported that *P. aeruginosa* *nemR* was induced by HOCl and
HOSCN, and *P. aeruginosa* *hypT* was induced by HOCl (25). Nevertheless, we demonstrate
consistencies in our results as *nemR*, *hypT*, and *ohrR* were neither HOCl-responsive, nor were
mutants in these genes HOCl-sensitive (Fig. S2). The reasons for these differences between the
Groitl et al study and ours are unclear, but they may be due to variations in the growth conditions
used, in particular the media used and the carbon sources metabolised.

Our study revealed that the bacterial response to HOCl and HOSCN is multifaceted and
incorporates an array of genes from different systems including metabolism, redox-sensing,
macromolecule repair and detoxification, export of toxic compounds and virulence. Figure 10
summarises the physiological processes described here as having putative roles in protecting *P.
aeruginosa* against HOCl and HOSCN in vitro and, we hypothesise, in the CF lung during
infection. Sulphur transporter and metabolism genes were upregulated following HOCl and
HOSCN exposure, and methionine metabolism regulators *SahR* and *MetR* appear required for
survival against HOCl (Fig. 3A and Fig. 8C). During sulphur starvation bacteria are able to use
alkanesulphonates and taurine as a sulphur source (59-62). Therefore, it is plausible that *P.
aeruginosa* requires increased uptake of alternative sulphur sources and careful control of
methionine metabolism to maintain levels of sulphur-containing compounds that are under
oxidative attack from HOCl and HOSCN. Upregulation of sulphur transport and metabolism, and
methionine and cysteine biosynthesis genes in response to HOCl stress has been reported in other
bacteria (12, 63-65). We found the catabolite repression control system (38) plays a role in
protection against HOCl; this indicates that appropriate regulation of carbon metabolism is
important for survival against HOCl (Fig. 3B-D). **RclR** upregulated expression of denitrification
genes in the presence of HOSCN (Fig. 9B). As our experiments were carried out aerobically we
would expect these Anr-regulated genes to be downregulated (66), therefore it suggests there may be a physiological advantage to expressing these pathways, aerobically in the presence of HOSCN.

Genes involved in repairing macromolecules and detoxification of oxidants were found to be important in the defence against HOCl and HOSCN. The H$_2$O$_2$-sensor OxyR and DNA repair enzyme RecG (42), were required for protection against HOCl (Fig. 4B). Non-coding tRNAs were upregulated in response to HOCl (Fig. 8A), perhaps to replace oxidatively damaged tRNA. Surprisingly, tRNA-Cys expression was unchanged in the presence of HOCl. RclR downregulated expression of 13 chaperone and heat shock genes, and upregulated expression of the chaperone gene cupA5, in the presence of HOSCN, but not HOCl (Fig. 9C). In the WT strain, expression of the chaperone genes was not markedly altered in the presence of HOCl nor HOSCN (Fig. 9C). In contrast, Groitl et al. found that HOCl and HOBr induced transcription of chaperone and heat shock genes, and together with HOSCN increased production of the chaperone polyphosphate, which protects *P. aeruginosa* against protein unfolding and aggregation caused by the oxidants (25). As previously mentioned, these differences may be due to variations in methodological approaches used in ours and the Groitl et al study, including the growth media and oxidant concentrations.

We found detoxification enzymes, in addition to RclX, were required for survival against HOCl, including the *pyeRM-xenB* operon, which encodes the oxidoreductase XenB (33) (Fig. 2C). The glutathione synthetase GshB was required for protection against HOCl (Fig. 4A); this enzyme is involved in synthesising glutathione, which maintains the redox potential of the cell in response to oxidative stress (12, 67). Previous transcriptome assays have highlighted the
importance of repair and detoxifying enzymes in the response of bacteria to HOCl stress (63-65, 68-71).

A striking finding of this work is a connection between the protective response to HOCl and HOSCN stress and antibiotic resistance. MexT and its regulated multidrug efflux pump MexEF-OprN were required for survival against HOCl (Fig. 2A). Additionally, the mexEF-oprN operon was upregulated in response to both HOCl and HOSCN (Fig. 8D), consistent with recent findings (25). While the fluoroquinolone, chloramphenicol and trimethoprim-exporting MexEF-OprN efflux pump is normally quiescent in WT cells or not expressed in strains with an inactive chromosomal copy of mexT, it is highly induced in nfxC type phenotypic mutants (mexT, mexS or mvaT) in a MexT dependent manner (72-73). This leads to increased resistance to fluoroquinolones and chloramphenicol and hypersensitivity to β-lactams (73). Of direct relevance to CF lung infections is the repeated observation that mutations in mexT occur in persistently infecting P. aeruginosa, both loss of function, as well as, gain of function mutations (nfxC-type) (74-77).

MexT has been shown previously to be a redox-responsive regulator modulating the response to electrophilic and nitrosative stress (78, 79). Our data extend the role of MexT and MexEF-OprN to protection of P. aeruginosa against HOCl and HOSCN stress and suggest that the cell has a pleiotropic need for induction of the MexEF-OprN efflux pump in response to a wide range of oxidative stress events. MexEF-OprN can expel products other than antibiotics, and increased expression of mexEF-oprN is associated with decreased quorum sensing due to efflux of quorum sensing molecules, specifically the PQS precursors, HHQ and kynurenine (80, 81). Therefore, one can propose that MexEF-OprN might additionally expel toxic by-products of HOCl and HOSCN reactions.
In conclusion, our findings have identified a wide range of different genes involved in the bacterial response to HOCl and HOSCN stress and provide the foundation from which further exploration of these mechanisms in protecting *P. aeruginosa* from these oxidants in the context of infection can be investigated.

MATERIALS AND METHODS

**Bacterial strains, plasmids and growth conditions**

All strains, plasmids and primers used in this study are listed in Table S1. Three anonymised clinical isolates of *P. aeruginosa* from airway secretions of infected CF patients were obtained from the Royal Brompton Hospital. PCR products and plasmids were sequenced by GENEWIZ, inc. Bacteria were routinely grown aerobically at 37°C, with shaking at 200 rpm or 700 rpm when in 96-well plates, in LB medium (5 g/L NaCl, 5 g/L yeast, 10 g/L tryptone) or on LBA plates (LB +1.5% (w/v) agar) supplemented with appropriate antibiotics. *P. aeruginosa* strains were isolated on *Pseudomonas* isolation agar (PIA) (Sigma) supplemented with 20% (v/v) glycerol. ASM was prepared as described by Kirchner, et al. however all individual amino acids, apart from L-tryptophan, were replaced with casamino acids, as described in Sriramulu, et al. (43, 44). The components of ASM per litre were: 4 g low molecular weight salmon sperm DNA, 5 g mucin from porcine stomach (type II), 4.75 g casamino acids, 0.25 g L-tryptophan, 5 g NaCl, 2.2 g KCl, 5.9 mg diethylenetriaminepentaacetic acid (DTPA), 5 ml egg yolk emulsion. The pH of ASM was adjusted to 6.9 and filter sterilised (44) and stored at 4°C for up to 1 month. After filtration the ASM slowly turns cloudy, due to precipitation of the salts from the media, but this does not impact its quality. Pyocyanin was extracted from 20-hour cultures grown in LB and concentration expressed in µg/ml as described in (82).
Construction of plasmids and mutant strains

Details of genes were obtained from The Pseudomonas Genome Database (27). In-frame deletions were constructed as previously described (83). Briefly, for construction of deletion mutants of PA14 *rclR*, *rclX*, *pyeRM*-xenB operon, and *oxyR* a ~500 bp upstream and ~500 bp downstream fragment of the genes was amplified by PCR using primer pairs 1F and 2R, and 3F and 4R (Table S1). Subsequent overlap PCR was performed using primers 1F and 4R (Table S1) to fuse the upstream and downstream fragments together to form a ‘mutator’ fragment that was purified and ligated into the pCR™-Blunt cloning vector (Invitrogen) for sequence-verification using M13F and M13R primers. The *rclR*, *rclX*, and *pyeRM*-xenB ‘mutator’ fragments were cloned into the BamHI site of the pKNG101 suicide vector and the *oxyR* ‘mutator’ fragment was cloned into the XbaI site of pKNG101, all were transformed into the donor CC118λpir *E. coli* strain and plated onto LBA supplemented with 50 µg/ml streptomycin. The *rclR*-pKNG101, *rclX*-pKNG101, *pyeRM*-xenB-pKNG101, and *oxyR*-pKNG101 plasmids from the donor strain were introduced into the PA14 recipient strain by conjugation. The DH5α *E. coli* strain containing the helper plasmid pRK2013 was grown on LBA supplemented with 50 µg/ml kanamycin and the recipient PA14 strain was grown on LBA. Colonies of the donor, helper and recipient strains were mixed together on LBA and incubated overnight. PA14 with the *rclR*-pKNG101, *rclX*-pKNG101, *pyeRM*-xenB-pKNG101, and *oxyR*-pKNG101 plasmids integrated site-specifically into the chromosome as single-crossovers were isolated on PIA with 2000 µg/ml streptomycin. Subsequently, for sucrose counter-selection isolated colonies were streaked onto LBA without NaCl and with 10% (w/v) sucrose and incubated at room temperature for 48 hours to obtain double-crossover unmarked in-frame deletion mutants. For *oxyR* deletion 100 U of
catalase from bovine liver per ml was added to the media. This is due to ΔoxyR mutants being unable to survive in LB, as components within the media autoxidise to generate ~ 1.2 µM H₂O₂ min⁻¹ (84). Colonies were isolated on LBA and deletion of rclR, rclX, pyeRM-xenB, and oxyR was confirmed by PCR and sequencing using 5F and 6R primers (Table S1). Subsequent initial overnight growth of ΔoxyR on LBA or in LB broth included 100 U/ml catalase. Complement plasmids, rclR-pUCP18 and rclX-pUCP18, were constructed by PCR amplification of the promoter region and ORF of PA14 rclR and rclX using primers in Table S1 and subsequent cloning into the HindIII/XbaI sites of the pUCP18 shuttle vector. The plasmids were transformed into DH5α E. coli and grown on LBA with 100 µg/ml ampicillin, prior to sequence verification using M13F and M13R primers (Table S1) and transformation into PA14 ΔrclR or ΔrclX mutant strains. The rclR-lacZ and rclX-lacZ plasmids were constructed by PCR amplification of the upstream promoter DNA (117 bp) of rclR and rclX using primers in Table S1 and ligation into pCR™-Blunt (Invitrogen) for sequence-verification using M13F and M13R primers. The promoter DNA of rclX and rclR was cloned into the EcoRI/PstI sites of the transcriptional fusion promoter-probe vector pMP220 (containing the lacZ gene) and transformed into DH5α E. coli and grown on LBA with 25 µg/ml tetracycline. Plasmids were sequence verified using primers Mid-PrcIR or Mid-PrcIX with Mid-lacZ (Table S1) prior to conjugation into PA14 WT and ΔrclR. Additionally, the rclX-lacZ plasmid was conjugated into three clinical CF isolates.

Preparation of HOCl and HOSCN solutions

Sodium hypochlorite solution with 10-15% available chlorine (Sigma) was diluted directly into LB or ASM to prepare the final HOCl concentrations required for assays. The concentration of HOCl was determined by diluting sodium hypochlorite into 40 mM potassium phosphate buffer.
(pH 7.5) with 10 mM sodium hydroxide and measuring the absorbance at 292 nm ($\varepsilon$ 350 M$^{-1}$ cm$^{-1}$) (25, 85). HOSCN was generated enzymatically by combining glucose, glucose oxidase (GO), potassium thiocyanate (KSCN) and LPO. GO catalyses the reaction of glucose with O$_2$ to form H$_2$O$_2$, which reacts with KSCN, catalysed by LPO, to form HOSCN. Stock concentrations of the enzymes 200 U/ml GO (Sigma) and 1000 U/ml LPO (Sigma) were prepared in 50% (v/v) PBS and 50% (v/v) glycerol solution and stored at -20°C. Stock concentrations of 4% (w/v) glucose and 750 mM KSCN (Fluka, 8M) were prepared in H$_2$O and stored at 4°C. Varying the amounts of glucose altered the amount of HOSCN produced and a final sublethal concentration of 0.01% glucose was selected for all assays performed in LB (apart from Fig. S4, for which a final amount of 0.02% glucose was used). The final concentrations of LPO, GO and KSCN were based on those used in (86). For the assays all components were diluted directly into LB or ASM to final concentrations of 0.01% glucose (0.02% glucose for Fig. S4), 0.5 U/ml GO, 0.75 mM KSCN and 3 U/ml LPO. A control assay without LPO that would only result in H$_2$O$_2$ generation (0.01% glucose, 0.5 U/ml GO and 0.75 mM KSCN) was performed to examine the effect of H$_2$O$_2$ (data not shown). The concentration of HOSCN was determined by reaction with 5-thio-2-nitrobenzoic acid (TNB), as described previously (25, 87). Briefly, 10 mM 5,5'-dithiobis (2-nitrobenzoic acid) (Thermo Scientific) prepared in 40 mM potassium phosphate buffer (pH 7.5) was hydrolysed by addition of sodium hydroxide to generate TNB. The TNB concentration was determined by measuring absorbance at 412 nm ($\varepsilon$ 14150 M$^{-1}$ cm$^{-1}$). HOSCN (0.01% glucose (0.02% glucose for Fig. S4), 0.5 U/ml GO, 0.75 mM KSCN and 3 U/ml LPO) prepared in 40 mM potassium phosphate buffer (pH 7.5) was diluted 1:1 in TNB and the loss of absorption at 412 nm was measured after 20 minutes. The concentration of HOSCN was determined from the concentration of TNB consumed during the reaction (stoichiometry 2:1, TNB: HOSCN). A
concentration of 0.8 mM HOSCN was produced from 0.01% glucose and 1 mM HOSCN from 0.02% glucose. For the ASM assays 0.8 mM HOSCN was produced, as described above, which was then diluted in ASM to give the final HOSCN concentrations used.

HOCl and HOSCN susceptibility and viability assays

A total of 707 strains with mutations in genes with regulatory functions were selected from the PA14 non-redundant single transposon mutant library (26) (Table S1). The strains were grown overnight in LB medium with 15 µg/ml gentamicin, alongside a PA14 WT control grown in LB medium, in 2.2 ml 96-well deep well plates (VWR). For the HOCl screen, overnight cultures were subcultured 1:20 into LB medium with 15 µg/ml gentamicin in 96-well microtitre plates (Falcon) and grown for 3 hours. Cultures were subcultured again 1:20 (optical density (OD) 0.05 ± 0.03) in LB medium or LB medium containing 4.4 mM HOCl in 96-well microtitre plates (Falcon) and grown for up to 24 hours with OD600 recorded as a measure of growth at hourly time points. LB medium was used for HOCl screening to facilitate growth of the large number of mutant strains, some of which may require nutrient-rich broth. The concentration of HOCl used was selected from a preliminary experiment, which identified 4.4 mM HOCl as being sub-lethal to PA14 WT; causing a lag in growth but not complete inhibition (data not shown). However, for the assays that tested the HOCl susceptibility of ΔpyeRM-xenB, 5.1 mM HOCl was used. For PA14 WT, ΔrclR and ΔrclX mutant strains, and ΔrclR+ rclR-pUCP18 and ΔrclX+ rclX-pUCP18 complemented strains, overnight cultures were prepared by growing the strains in LB medium or LB medium with 500 µg/ml carbenicillin for the pUCP18 complemented strains. Overnight cultures were subcultured 1:20 in LB medium and grown for 3 hours, prior to subculturing again 1:20 (OD 0.05 ± 0.02) into LB medium or LB medium with 4.4 mM HOCl or 0.8 mM HOSCN.
and following growth by recording OD_{600}. The concentration of HOSCN was chosen from initial growth curve analyses, which identified 0.8 mM and 1mM HOSCN as sub-inhibitory for ΔrcIR and ΔrcIX, respectively (data not shown). However as the higher concentration was lethal to ΔrcIR, the lower concentration of 0.8 mM was used for all experiments performed in LB, apart from Fig. S4. For the HOCl and HOSCN susceptibility assays in ASM media, the PA14 WT, ΔrcIR and ΔrcIX mutant strains were grown as above. The only difference was the ASM was filtrated again prior to adding HOCl at a final concentration of 3.1 mM, and adding components to make 0.8 mM HOSCN, which was diluted to a final concentration of 0.53 mM. Lower concentrations were required in ASM, as bacteria had increased susceptible to both oxidants in this media. For HOCl and HOSCN viability assays, cultures were sampled at 0 hour and 2.5 hour or 4.5 hour after HOSCN or HOCl exposure, respectively, and 10-fold serial dilutions in PBS were prepared and dilutions were drop plated on LBA. For other chemical susceptibility assays, PA14 WT, ΔrcIR and ΔrcIX strains were grown as before, but in LB medium containing 0.125 mM NEM, 5 mM diamide, 2.5 mM methylglyoxal, 5 mM H_2O_2, 0.5 mM TBH, 1 mM methyl viologen or 10 mM DEANO. The concentrations of chemicals used were determined from preliminary experiments that tested a range of concentrations to identify those that caused a lag, but not inhibition, in growth of PA14 WT (data not shown). All chemicals were from Sigma apart from TBH that was from EMD Millipore. For ASM biofilm assays we adapted the method from (44). Overnight cultures of PA14 WT, ΔrcIR and ΔrcIX, were subcultured 1:50 in LB and grown for 3 hours, prior to subculturing again 1:50 into 2ml ASM without or with HOCl (3.1 or 3.5 mM) and HOSCN (0.43 mM or 0.53 mM) in a 24-well tissue culture treated plate (Falcon), which was incubated at 37 °C, low shaking, for 3 days to allow biofilm formation, and then left at room temperature, static, for a further 4 days before visualisation.
RNA extraction and RNA-seq analysis

Exponential phase PA14 WT and ΔrcIR cultures were subcultured 1:50 in LB (OD 0.05 ± 0.02) and grown for 2.5 hours prior to adding HOCl to a final concentration of 2.2 mM, or HOSCN to a final concentration of 0.8 mM, or no treatment (LB only). LB medium was used for consistency with the HOCl screen. The concentrations of oxidants used were chosen due to causing a 30-120 minute lag in growth of exponential phase cultures. Treated and untreated WT and ΔrcIR cultures were incubated for a further 20 minutes and then cells were collected and centrifuged at 13000 x g for 5 minutes. The pellets were washed twice with PBS and RNAlater (Ambion) was added to preserve the RNA. Two biological replicates of WT and ΔrcIR treated with HOCl or HOSCN or untreated, from independent experiments, were collected for RNA purification. Enzymatic lysis and proteinase K digestion of bacteria followed by RNA purification was carried out using the RNeasy Protect Bacteria Mini Kit (QIAGEN). DNase treatment of RNA samples was performed using the TURBO DNA-free kit (Ambion). Removal of rRNA, preparation of cDNA libraries and sequencing using the Illumina NextSeq 500 system and a 75 bp read length was performed by vertis Biotechnologie AG. Reads were aligned to the *P. aeruginosa* UCBPP-PA14, complete genome (NCBI accession number: NC_008463.1) using bowtie2 and expression values in Reads Per Kilobase per Million mapped reads (RPKM) were calculated using FeatureCounts. Normalisation of the RPKM values against the OD_{600} of the samples prior to RNA extraction was performed and the values of the replicates were averaged. The log2 fold change of the RPKM values of WT treated compared to WT untreated or WT treated compared to ΔrcIR treated was calculated. Genes with a log2 fold change of >1.5 and <-1.5 were considered to be differentially expressed.
Quantitative real-time PCR

Cultures of PA14 WT were grown to mid-exponential phase in LB and treated with HOCl or HOSCN for 20 minutes prior to RNA extraction, this was performed as described for RNA_seq. The cDNA library was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was set up with Fast SYBR Green mix (Invitrogen) and using the Applied Biosystems™ 7500 Fast Real-Time PCR Instrument. Expression ratios were calculated comparing the expression of each gene in PA14 WT treated cultures to PA14 WT untreated, by the ∆∆CT method (88) and normalised to expression of rpoD (RNA polymerase sigma factor), which shows unchanged expression levels under the conditions tested. Primers used for qRT-PCR are listed in Table S1.

β-Galactosidase assays

PA14 WT and ΔrclR strains containing rclR-lacZ and rclX-lacZ plasmids, and clinical CF isolates 1, 2 and 3 containing the rclX-lacZ plasmid were grown in LB medium with 100 µg/ml tetracycline overnight. Cultures were subcultured 1:50 (OD 0.05 ± 0.02) in LB medium in 2.2 ml 96-well deep well plates (VWR) and grown to exponential phase (3 hours) and were then either untreated or treated with 2.2 mM HOCl or 0.8 mM HOSCN. Cultures were collected 30 minutes after treatment with HOCl or HOSCN. β-Galactosidase activity was assayed using the modified version (89) of the Miller method (90).

Homology modelling and sequence analysis
The predicted structure of RclX was modelled with SWISS-MODEL (91) and illustrations of protein structures were prepared with EzMol (92). Amino acid sequences were aligned using Clustal Omega (93) and visualised with ESPript 3.0 (94).

**Data availability**

The transcriptomic data of our RNAseq experiments have been deposited in the NCBI’s GEO database (95) under accession number GSE124385 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124385).

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FIGURE LEGENDS

FIG 1 Identification of PA14 regulatory gene mutants with altered susceptibility to HOCl. Example screening plate with HOCl-sensitive (mexT and PA14_07110 (sahR)) and HOCl-resistant (PA14_53410 (1)) mutants labelled. Strains were grown in a 96-well format in LB medium with 4.4 mM HOCl and optical density (OD$_{600}$) was recorded as a measure of growth. Mutant strains that had an increased lag >3 hours compared to WT were identified as HOCl-sensitive and strains with a decreased lag >3 hours compared to WT were identified as HOCl-resistant. Arrow represents the end of WT lag phase. Strains grown in the absence of HOCl showed minor variations in growth, but none of the strains selected as sensitive or resistant (Table 1 and 2) had growth defects in LB only (data not shown).

FIG 2 Mutants of genes involved in antimicrobial resistance displayed altered susceptibility to HOCl. (A) Gene arrangement of mexT and its adjacent mexEF-oprN operon. Growth of PA14 WT, mexT, mexE, mexF, and oprN strains in the presence of 4.4 mM HOCl. (B) Gene
arrangement of \( nfxB \) and its adjacent \( mexCD-oprJ \) operon. Growth of PA14 WT, \( nfxB \), \( mexC \), \( mexD \) and \( oprJ \) strains in the presence of 4.4 mM HOCl. For \( mexC \) and \( mexD \), 2 transposon mutants of each gene were available and showed similar phenotypes, therefore only 1 mutant of each is displayed. (C) Gene arrangement of the \( pyeRM-xenB \) operon; PyeR autorepresses the \( pyeRM-xenB \) operon (33). Growth of PA14 WT and \( \Delta pyeRM-xenB \) strains in the presence of 5.1 mM HOCl. Graphs display the means and standard error of the means for (A+B) three independent experiments and (C) three biological replicates, representative of three independent experiments. The (+) and (-) signs in the gene arrangement diagrams represent positive and negative regulators, respectively. All mutant strains displayed statistically significant altered susceptibility to HOCl compared with WT at different time points, apart from \( oprN \), \( mexC \), \( mexD \) and \( oprJ \) (\( p<0.05 \); one-way ANOVA with Dunnett’s multiple comparisons test). Strains grown in the absence of HOCl showed no growth defects (data not shown). All transposon mutants and the in-frame deletion were confirmed by PCR.

FIG 3 Mutants of genes involved in metabolism displayed altered susceptibility to HOCl. (A) Growth of PA14 WT, \( metR \) and PA14_07110 (\( sahR \)) strains in the presence of 4.4 mM HOCl. (B) Gene arrangement of the two-component system \( cbrAB \) that regulates expression of the sRNA \( CrcZ \), which relieves Crc/Hfq-mediated repression of target mRNAs. (C) Growth of PA14 WT, \( cbrA \) and \( cbrB \) strains and (D) PA14 WT, and \( crc \) strains, in the presence of 4.4 mM HOCl. For \( cbrA \), 2 transposon mutants were available and showed similar phenotypes, therefore only 1 mutant is displayed. Graphs display the means and standard error of the means for (A+C) three independent experiments and (D) three biological replicates, representative of three independent experiments. The (+) sign in the gene arrangement diagram represents a positive regulator. All
mutant strains displayed statistically significant altered susceptibility to HOCl compared with WT at different time points, ($p<0.05$; one-way ANOVA with Dunnett’s multiple comparisons test). Strains grown in the absence of HOCl showed no growth defects (data not shown). All transposon mutants were confirmed by PCR.

**FIG 4** Mutants of genes involved in oxidative stress defence displayed altered susceptibility to HOCl. (A) Gene arrangement of the predicted 4-gene operon, gshB-tonB3-algH-yqgF. Growth of PA14 WT, algH and gshB strains in the presence of 4.4 mM HOCl. PA14 transposon mutants were not available for yqgF but were for tonB3, however they did not display altered HOCl susceptibility (data not shown). (B) Gene arrangement of the oxyR-recG operon. Growth of PA14 WT, recG and ΔoxyR strains in the presence of 4.4 mM HOCl. (C) Gene arrangement of PA14_07340 (rclR) and PA14_07355 (rclX). Growth of PA14 WT, PA14_07340 (rclR) and PA14_07355 (rclX) strains in the presence of 4.4 mM HOCl. Graphs display the means and standard error of the means for three independent experiments (two independent experiments only for recG). All mutant strains displayed statistically significant altered susceptibility to HOCl compared with WT at different time points, ($p<0.05$; one-way ANOVA with Dunnett’s multiple comparisons test). Strains grown in the absence of HOCl showed no growth defects (data not shown). All transposon mutants and the in-frame deletion were confirmed by PCR.

**FIG 5** Mutants of rclR and its adjacent gene rclX are sensitive to HOCl, and rclR is sensitive to the epithelial-derived oxidant HOSCN. (A-C) Growth of PA14 WT, ΔrclR, ΔrclR + rclR-pUCP18, ΔrclX, ΔrclX + rclX-pUCP18 strains in (A) LB medium (B) LB medium with 4.4 mM HOCl or (C) LB medium with 0.8 mM HOSCN. Graphs display the means and standard error of
the means for three biological replicates, representative of three independent experiments. For HOSCN-sensitivity assays a control reaction of glucose oxidase, glucose and potassium thiocyanate was carried out, which resulted in production of H$_2$O$_2$ only; $\Delta rclR$ was not sensitive under these conditions confirming sensitivity is due to HOSCN production (data not shown). Addition of the empty pUCP18 vector to $\Delta rclR$ and $\Delta rclX$ had no effect on the susceptibility of these mutants to HOCI or HOSCN (data not shown). (D-E) Viability CFU assays, mid-exponential phase cultures of PA14 WT, $\Delta rclR$, $\Delta rclX$, $\Delta rclR + rclR$-pUCP18 and $\Delta rclX + rclX$-pUCP18 were incubated in LB medium containing (D) 4.4 mM HOCI or (E) 0.8 mM HOSCN. Samples were taken immediately after oxidant exposure and after 4.5 hours (HOCI) or 2.5 hours (HOSCN), and diluted in PBS and spot-titrated onto LBA and incubated at 37°C overnight or until CFUs could be visualised. For undiluted ($10^{10}$) $\Delta rclR$ samples taken immediately after HOSCN exposure (0 hour) (E), fewer viable cells are visualized because HOSCN acted immediately, whereas in the other dilution factors HOSCN was diluted down and therefore did not impact growth. Images are representative of three independent experiments.

**FIG 6** HOCI and HOSCN stress induces RclR-dependent regulation of $rclX$ expression in PA14 *P. aeruginosa* and clinical CF isolates. (A) Activity of $rclR$-lacZ and $rclX$-lacZ transcriptional fusions in the PA14 WT and $\Delta rclR$ strains in the absence or presence of 2.2 mM HOCI or 0.8 mM HOSCN. (B) Activity of the $rclX$-lacZ transcriptional fusions in WT, $\Delta rclR$, and clinical CF isolates 1, 2 and 3 in the absence or presence of 2.2 mM HOCI or 0.8 mM HOSCN. Strains were grown aerobically in LB medium until mid-exponential phase when HOCI or HOSCN or LB only was added and samples were taken for $\beta$-galactosidase activity measurements at 30 minutes. The concentrations of oxidants used caused a 30-120 minute lag in growth of exponential phase
cultures. PA14 WT and ΔrclR carrying the empty pMP220 vector expressed low β-galactosidase activity (~100 Miller Units) under all of the treatment conditions (data not shown). Data show the means and standard error of the means for three biological replicates, representative of three independent experiments (six biological replicates for (B) untreated strains). Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons post-hoc test. ** indicates p<0.01 and **** indicates p<0.0001, compared to the same strain untreated (no oxidant).

FIG 7 Relative changes in gene expression in PA14 WT and ΔrclR in the presence of HOCl or HOSCN. Log2 fold change of gene expression plotted against the gene locus tag in order of genomic location for (A) Effect of HOCl on *P. aeruginosa* gene expression: WT+ HOCl vs. WT untreated, (B) Effect of HOSCN on *P. aeruginosa* gene expression: WT+ HOSCN vs. WT untreated, (C) Identification of genes regulated by RclR in the presence of HOCl: WT+ HOCl vs. ΔrclR+ HOCl or (D) Identification of genes regulated by RclR in the presence of HOSCN: WT+ HOSCN vs. ΔrclR+ HOSCN. Lines indicate cut off values at 1.5 or -1.5 log2 fold change. (A+B) Highlighted upregulated genes: *rclX*, hypothetical operon (PA14_21570-PA14_21580-PA14_21590-PA14_21600), sulphur transport genes (*ssuD*-PA14_19570-*ssuB*), taurine transport genes (PA14_12920-PA14_12940-PA14_12960), drug efflux pump operon *mexEF-oprN*, *pyeRM-xenB* operon (HOCl), and T3SS genes; downregulated genes: *pyeRM* (HOSCN). (C+D) In these plots positive log2 fold change values indicate genes positively regulated by RclR and negative log2 fold change values indicate genes negatively regulated by RclR. Highlighted genes positively regulated: *rclX*, pyocyanin biosynthesis operons *phz1* and *phz2*, denitrification *nir* and
nar operons (HOSCN), and pyeRM (HOCl); negatively regulated: pyeRM-xenB (HOSCN), mexEF-oprN, T3SS genes (HOSCN), and heat shock protein ibpA (HOSCN).

FIG 8 Heat maps displaying expression of tRNA, T3SS, sulphur and taurine transport and metabolism, and mexEF-oprN efflux pump and pyeRM-xenB genes in PA14 WT and ΔrclR after HOCl or HOSCN exposure. (A) tRNAs, (B) T3SS, (C) sulphur and taurine transport and metabolism and (D) mexEF-oprN and pyeRM-xenB. Expression of genes is colour coordinated from 6.5 log2 fold change (blue) to -6.5 log2 fold change (yellow). The first column indicates HOCl responsive genes (log2 fold change of WT+ HOCl vs. WT untreated (UT)) and the second column indicates HOSCN responsive genes (log2 fold change of WT+ HOSCN vs. WT UT). The third column indicates RclR-regulated genes under HOCl stress (log2 fold change of WT+ HOCl vs. ΔrclR+ HOCl) and the fourth column indicates RclR-regulated genes under HOSCN stress (log2 fold change of WT+ HOSCN vs. ΔrclR+ HOSCN), and in these columns, genes that are positively or negatively regulated are indicated by blue or yellow, respectively. Broken lines indicate the start and end of operons.

FIG 9 Heat maps displaying expression of pyocyanin biosynthesis, denitrifying enzyme, and chaperone and heat shock genes in PA14 WT and ΔrclR after HOCl or HOSCN exposure. (A) pyocyanin biosynthesis and other virulence factors, (B) denitrifying enzymes and (C) chaperones (genes in bold indicate the chaperones). Expression of genes is colour coordinated from 6.5 log2 fold change (blue) to -6.5 log2 fold change (yellow). The first column indicates HOCl responsive genes (log2 fold change of WT+ HOCl vs. WT UT) and the second column indicates HOSCN responsive genes (log2 fold change of WT+ HOSCN vs. WT UT). The third column indicates...
RclR-regulated genes under HOCl stress (log2 fold change of WT+ HOCl vs. ∆rclR+ HOCl) and the fourth column indicates RclR-regulated genes under HOSCN stress (log2 fold change of WT+ HOSCN vs. ∆rclR+ HOSCN), and in these columns, genes that are positively or negatively regulated are indicated by blue or yellow, respectively. Broken lines indicate the start and end of operons.

FIG 10 Model of the putative defence mechanisms used by *P. aeruginosa* in response to HOCl and HOSCN stress. 1. *P. aeruginosa* responds to HOCl and HOSCN by upregulation of sulphur transport and metabolism genes; this may facilitate increased uptake of alternative sulphur sources, including taurine, to replenish depleted sulphur levels caused by HOCl and HOSCN oxidative damage. Methionine biosynthesis and metabolism regulators MetR and SahR appear required to protect against HOCl, which we postulate is a response to oxidative disruption of the metabolism of the sulphur-containing amino acid methionine and the cellular need to maintain its levels. Catabolite repression by the Crc protein appears required for protection against HOCl, possibly indicating that optimal regulation of metabolic flexibility aids defence against HOCl. 2. The transcriptional regulator RclR, the H$_2$O$_2$-sensor OxyR (42) and the hypothetical protein AlgH (41), all have putative redox-sensing capabilities and were identified as playing a role in protection against HOCl. 3. Macromolecular repair mechanisms are implicated in the response to HOCl and HOSCN. Upregulation of tRNAs in response to HOCl may occur to replace oxidatively damaged tRNA. The DNA repair enzyme RecG is required for protection against HOCl. 4. Putative bacterial detoxification enzymes are involved in the response to HOCl and HOSCN. The PyeR-regulated oxidoreductase XenB is induced in response to HOCl and is required for protection and the glutathione synthetase GshB is required for protection against
HOCl. The peroxiredoxin RclX is positively regulated by RclR in response to HOCl and HOSCN and required for protection against both oxidants. 5. Upregulation of the MexT-regulated mexEF-oprN operon, encoding a multidrug efflux pump (28, 29) occurs in response to HOCl and HOSCN and is required for protection against HOCl. This highlights a link between oxidative stress and antibiotic resistance, and possibly indicates a role for the efflux pump in expelling toxic by-products of these oxidants. 6. RclR is required for survival against HOCl and HOSCN and regulates a number of genes in the presence of both oxidants, including upregulation of the phz genes which synthesise the virulence factor pyocyanin (52). The genes responsible for the biogenesis and regulation of another virulence mechanism, the T3SS (51), are upregulated in response to HOCl and HOSCN. Induction of pyocyanin and the T3SS may occur as a mechanism to counterattack the innate immune cell-derived HOCl and HOSCN-mediated oxidative attack on the bacteria.
TABLE 1 HOCl-sensitive regulatory gene mutants

| PA14 Locus Tag/ Gene Name<sup>a</sup> | PAO1 Locus Tag | Protein Description | One-way ANOVA with Dunnett’s multiple comparisons test, p value | Mutant OD<sub>600</sub> vs WT OD<sub>600</sub> at set time points<sup>b</sup> |
|--------------------------------------|----------------|---------------------|-------------------------------------------------------------|-------------------------------------------------------------|
|                                      |                |                     | 20hr                                                 | 22hr                                                      | 24hr            |
| PA14_07110 sahR                       | PA0547         | ArsR-family transcriptional regulator | 0.0420                                                 | 0.0003                                                    | <0.0001        |
| PA14_08780 rpoC                       | PA4269         | DNA-directed RNA polymerase β chain | 0.0280                                                 | 0.0002                                                    | <0.0001        |
| PA14_13660 tpbA                       | PA3885         | Protein tyrosine phosphatase | 0.0522                                                 | 0.0006                                                    | 0.0005          |
| PA14_17900 metR                       | PA3587         | LysR-family transcriptional regulator | 0.0342                                                 | 0.0002                                                    | <0.0001        |
| PA14_20730 flagM (1)                  | PA3351         | Flagellar biosynthesis anti-sigma-28 factor | 0.0288                                                 | 0.0002                                                    | <0.0001        |
| PA14_20730 flagM (2)                  | PA3351         | Flagellar biosynthesis anti-sigma-28 factor | 0.0299                                                 | 0.0001                                                    | <0.0001        |
| PA14_22370 (2)                        | PA3233         | Hypothetical protein, cyclic nucleotide-binding domain | 0.0274                                                 | 0.0002                                                    | <0.0001        |
| PA14_29740                            | PA2656         | Two-component sensor | 0.2349                                                 | 0.0427                                                    | 0.0214          |
| PA14_32410 mexT                        | PA2492         | LysR-family transcriptional regulator of multidrug efflux | 0.0370                                                 | 0.0002                                                    | <0.0001        |
| PA14_45950 rsaL.                      | PA1431         | Regulatory protein | 0.1666                                                 | 0.0367                                                    | 0.0385          |
| PA14_52570 rsmA                       | PA0905         | Posttranscriptional global regulator | 0.0340                                                 | 0.0003                                                    | <0.0001        |
| PA14_56620 pyeR (2)                   | PA4354         | ArsR-family transcriptional regulator | 0.0396                                                 | 0.0010                                                    | 0.0068          |
| PA14_60860 nfxB                       | PA4600         | TetR-family transcriptional regulator of multidrug efflux | 0.0515                                                 | 0.0049                                                    | 0.0235          |
| PA14_62490 dksA                       | PA4723         | RNA polymerase-binding transcription factor | 0.0088                                                 | 0.0006                                                    | 0.0018          |
| PA14_68680 envZ (3)                   | PA5199         | Two-component sensor | 0.0494                                                 | 0.0082                                                    | 0.0714          |
| PA14_70570 recG<sup>c</sup>           | PA5345         | ATP-dependent DNA helicase | 0.0053                                                 | <0.0001                                                   | <0.0001        |

<sup>a</sup>Mutants were identified as HOCl-sensitive by visual inspection (increased lag >3 hour compared to WT) and statistical analysis of growth. <sup>b</sup>Statistical analysis performed on two biological replicates. <sup>c</sup>recG is not a regulatory mutant per se, but is on the same operon as oxyR that encodes the H<sub>2</sub>O<sub>2</sub>-sensing transcriptional regulator; the transposon mutant in oxyR was not
recoverable. The bracketed numbers next to some of the gene mutants, correspond to genes that have more than one mutant strain in the transposon library (Table S1).

**TABLE 2** HOCl-resistant regulatory gene mutants

| PA14 Locus Tag/ Gene Name<sup>a</sup> | PA01 Locus Tag | Description | One-way ANOVA with Dunnett’s multiple comparisons test, p value<sup>b</sup> |
|-------------------------------------|----------------|-------------|-----------------------------------------------|
| PA14_06950 (1)                      | PA0533         | LuxR-family transcriptional regulator         | Mutant OD<sub>600</sub> vs WT OD<sub>600</sub> at set time points |
| PA14_09760                          | PA4185         | GntR-family transcriptional regulator        | <0.0001          | <0.0001          | <0.0001          | <0.0001          |
| PA14_10800 <i>ampR</i>              | PA4109         | LysR-family transcriptional regulator of β-lactamase | 0.9538          | 0.0098          | 0.0170          | 0.2154          |
| PA14_27900                          | PA2802         | GntR-family transcriptional regulator        | <0.0001          | <0.0001          | <0.0001          | 0.0003          |
| PA14_38040 <i>cmrA</i>              | PA2047         | AraC-family transcriptional regulator         | 0.1181          | 0.0040          | 0.0040          | 0.0442          |
| PA14_44490 <i>anr</i>               | PA1544         | Transcriptional regulator of anaerobic metabolism | <0.0001          | <0.0001          | <0.0001          | <0.0001          |
| PA14_50180 <i>fleR</i> (1)          | PA1099         | Two-component response regulator of flagellar biosynthesis | 0.9990          | 0.2376          | 0.0471          | 0.3638          |
| PA14_50200 <i>fleS</i> (2)          | PA1098         | Two-component sensor of flagellar biosynthesis | 0.0610          | <0.0001          | 0.0017          | 0.0481          |
| PA14_56620 <i>pyeR</i> (1)          | PA4354         | ArsR-family transcriptional regulator         | <0.0001          | <0.0001          | <0.0001          | <0.0001          |
| PA14_62530 <i>cbrA</i> (1)          | PA4725         | Two-component sensor of catabolic pathways   | <0.0001          | <0.0001          | <0.0001          | 0.0398          |
| PA14_62530 <i>cbrA</i> (2)          | PA4725         | Two-component sensor of catabolic pathways   | 0.8620          | 0.0482          | 0.0405          | 0.3687          |
| PA14_62540 <i>cbrB</i>              | PA4726         | Two-component response regulator of catabolic pathways | 0.0049          | 0.0003          | 0.0140          | 0.2098          |
| PA14_68680 <i>envZ</i> (1)          | PA5199         | Two-component sensor                         | <0.0001          | <0.0001          | <0.0001          | 0.0015          |
Mutants were identified as HOCl-resistant by visual inspection (decreased lag >3 hour compared to WT) and statistical analysis of growth. Statistical analysis performed on two biological replicates. The bracketed numbers next to some of the gene mutants, correspond to genes that have more than one mutant strain in the transposon library (Table S1).
A

![Figure A showing 
\[ \text{\beta-galactosidase activity (Miller Units)} \]

- **WT + rclR-lacZ**
- **ΔrcIR + rclR-lacZ**
- **WT + rclX-lacZ**
- **ΔrcIR + rclX-lacZ**

No oxidant | + HOCl | + HOSCN
---|---|---

B

![Figure B showing 
\[ \text{\beta-galactosidase activity (Miller Units)} \]

- **WT + rclX-lacZ**
- **ΔrcIR + rclX-lacZ**
- **Clinical 1 + rclX-lacZ**
- **Clinical 2 + rclX-lacZ**
- **Clinical 3 + rclX-lacZ**

No oxidant | + HOCl | + HOSCN
