**Pseudomonas aeruginosa** encoding tRNA-thiolating protein requires an iron-sulfur cluster to participate in hydrogen peroxide-mediated stress protection and pathogenicity

Adisak Romsang1,2, Jintana Duang-nkern3, Khwannarin Khemsom1, Lampet Wongsaroj4, Kritsakorn Saninjuk1, Mayuree Fuangthong3, Paiboon Vattanaviboon3 & Skorn Mongkolsuk1,2,3,4

During the translation process, transfer RNA (tRNA) carries amino acids to ribosomes for protein synthesis. Each codon of mRNA is recognized by a specific tRNA, and enzyme-catalysed modifications to tRNA regulate translation. TtcA is a unique tRNA-thiolating enzyme that requires an iron-sulfur ([Fe-S]) cluster to catalyse thiolation of tRNA. In this study, the physiological functions of a putative ttcA in *Pseudomonas aeruginosa*, an opportunistic human pathogen that causes serious problems in hospitals, were characterized. A *P. aeruginosa* ttcA-deleted mutant was constructed, and mutant cells were rendered hypersensitive to oxidative stress, such as hydrogen peroxide (H2O2) treatment. Catalase activity was lower in the ttcA mutant, suggesting that this gene plays a role in protecting against oxidative stress. Moreover, the ttcA mutant demonstrated attenuated virulence in a *Drosophila melanogaster* host model. Site-directed mutagenesis analysis revealed that the conserved cysteine motifs involved in [Fe-S] cluster ligation were required for TtcA function. Furthermore, ttcA expression increased upon H2O2 exposure, implying that enzyme levels are induced under stress conditions. Overall, the data suggest that *P. aeruginosa* ttcA plays a critical role in protecting against oxidative stress via catalase activity and is required for successful bacterial infection of the host.

The ability of pathogenic bacteria to successfully invade a host is largely associated with their ability to rapidly adapt to and overcome host immune systems. Reactive oxygen species (ROS) are reactive molecules and free radicals derived from the incomplete reduction of oxygen. ROS are sequentially produced by the electron transport pathway during aerobic respiration by phagolysosomes in phagocytic cells, which facilitate attacks on invading microbes1,2. ROS also play roles in cellular signalling pathways, including apoptosis, necrosis, gene expression, and the activation of cell signalling cascades3. An imbalance between the production and removal of ROS (excess ROS) is referred to as oxidative stress, which causes damage to nucleic acids, lipid peroxidation, protein oxidation, enzyme inhibition, and cofactor inactivation4. Accordingly, pathogens have evolved mechanisms to protect themselves against host-generated stresses by scavenging excess ROS with cellular enzymes, such as superoxide dismutase (Sod) and catalase (Kat), and the rebuilding of cofactors such as iron-sulfur clusters ([Fe-S]) via the ISC system along with the repair of oxidative damaged proteins via the methionine sulfoxide reductase (Msr) system5–7. To attain the highest efficiency and execute successful infection, the complex processes...
underlying bacterial sensing and responses to stress are controlled by specific mechanisms carried out by various transcriptional regulators. For example, OxyR, a LysR-type transcriptional regulator, is a global stress response protein involved in hydrogen peroxide (H₂O₂) defence via the activation of genes encoding Kat, while SoxR, a [2Fe-2S] cluster-containing transcription factor, triggers a major response to redox active compounds by activating antioxidant-encoding genes including sod. The mechanisms required for adaptive responses to such stresses primarily involve transcriptional controls; however, some bacteria also exhibit adaptive mechanisms for post-transcriptional regulation.

Translational controls in prokaryotes usually involve modifications to tRNA, which is a key molecule for protein synthesis with multiple points of stress-induced regulation. tRNA modifications are catalysed by an enzyme with the potential to influence specific anticodon-codon interactions and regulate translation. A previous study described specific transcripts with particular codon biases encoding stress response proteins that are translationally regulated by dynamic changes in tRNA wobble base modifications. Numerous enzymes have been identified in modification pathways for bacterial tRNAs, such as GidA/MmmE (involved in bacterial virulence in several pathogenic bacteria) and TrmJ (functions in the oxidative stress response in Pseudomonas aeruginosa). Escherichia coli TtcA, a 2-thiocytidine tRNA biosynthesis protein, catalyses the post-transcriptional thiolation of cytosine 32 as s²C32 in some tRNAs. TtcA contains a redox-active and oxygen-sensitive [4Fe-4S] cluster that is chelated by cysteine residues and is absolutely essential for activity. The modified nucleoside s²C32 has thus far been found in tRNAs from organisms belonging to the Archaeal and Bacterial domains. The TtcA protein family is characterized by the presence of both a PP-loop and a Cys-X-X-Cys motif in the central region of the protein but can be divided into two distinct groups based on the presence of additional Cys-X-Cys motifs in terminal regions of the protein sequence. Mutant analysis in E. coli showed that both cysteine residues in this central conserved Cys-X-Cys motif are required for the formation of s²C32. The biochemical mechanisms of TtcA that catalyses the thiolation of cytosine 32 have been well studied; however, the physiological function of this enzyme has never been reported.

Pseudomonas aeruginosa is one of the most common opportunistic human pathogens and causes lethal infections in patients with impaired immune systems or in critical condition. Hospital-acquired infections caused by P. aeruginosa are increasing with global epidemiology. Expanding our knowledge of the regulatory virulence network in this bacterium will facilitate the identification of potential drug targets. In this study, P. aeruginosa ttcA encoding TtcA, which contains conserved Cys-X-Cys motifs to bind the [Fe-S] cluster, was functionally characterized in response to oxidative stress and was found to play a role in the pathogenicity of this bacterium.

Results and Discussion

Identification of ttcA in P. aeruginosa. The P. aeruginosa PAO1 genome contains the 825-bp open reading frame (ORF) PA1192, annotated as a conserved hypothetical gene encoding a protein with high homology to E. coli TtcA, a tRNA 2-thiocytidine biosynthesis protein. P. aeruginosa PA1192 has a theoretical molecular mass of 31.3 kDa, and its deduced amino acid sequence shares 67.2% and 66.8% sequence identity with TtcA from Escherichia coli and Salmonella enterica serovar Typhimurium, respectively (Fig. 1a). No paralogous gene of PA1192 in the PAO1 genome was found. The TtcA signature motif (LGGKDKS) in the PP-loop family as well as the iron-sulfur cluster binding domains Cys-X-X-Cys (C115-S-L-C118) and Cys-X-Cys (C203-N-L-C206) are conserved in P. aeruginosa PA1192 (Fig. 1a). In this study, P. aeruginosa PA1192 was annotated as a putative ttcA and further noted as ttcA.

P. aeruginosa ttcA is located 47 bp upstream of PA1193, a hypothetical protein (Fig. 1b). Analysis of the transcriptional organization of these genes by Northern blotting and RT-PCR using primers located in the ttcA and PA1193 genes indicated that they are transcribed separately (Fig. 1c). ttcA is arranged 102 bp apart from PA1191, a hypothetical protein partially containing a putative DnaJ-homologous sequence, in the opposite strand (Fig. 1b).

Purified TtcA binds an oxidant-sensitive iron-sulfur cluster. To detect iron-sulfur cluster-TtcA ligation, P. aeruginosa TtcA expression in Escherichia coli and TtcA protein purification were performed as described in the Methods. The purified TtcA was then subjected to UV-visible spectroscopy scanning analysis to determine the presence of iron-sulfur clusters. The results in Fig. 2a show significant absorption at 420 nm in the UV-visible spectrum of the purified TtcA, suggesting the presence of a [Fe-S] cluster ligated with the protein, similar to the results of previous studies investigating the characteristics of iron-sulfur cluster proteins. This finding was supported by an in silico analysis of the iron-sulfur cluster binding domains in the P. aeruginosa TtcA sequence, which contained two separate Cys-X-X-Cys motifs indicative of [Fe-S] cluster ligation.

Oxidative damage occurs when ROS oxidize an exposed Fe²⁺ atom in the [4Fe-4S] cluster through a metal-based oxidation mechanism, resulting in the ejection of an iron atom from the cluster and subsequent reduction of the cluster to the inactive [3Fe-4S] oxidation state. To determine the effects of H₂O₂ on [Fe-S] cluster integrity, purified TtcA was incubated with various concentrations of H₂O₂ prior to performing UV-visible spectroscopy. The results showed decreases in TtcA absorbance at 420 nm that were H₂O₂ concentration-dependent (Fig. 2a), suggesting that ligation of the [Fe-S] cluster to TtcA provided targets for H₂O₂-mediated oxidation (5–50 mM), resulting in the destabilization of iron-sulfur clusters bound to the protein. Treatment of the protein with a high concentration (0.5 M) of H₂O₂ led to the total loss of [Fe-S] clusters bound to TtcA, as shown in Fig. 2b. Together with the previously described results, we found that P. aeruginosa TtcA contains the ROS-sensitive [Fe-S] cluster as its cofactor, similar to TtcA in E. coli, which contributes to the thiolation of cytosine 32 in tRNA, however, the importance of this cofactor for extended physiological function, particularly under oxidative stress conditions, still needs to be further investigated.

The ΔttcA mutant shows increased susceptibility to H₂O₂ and sodium hypochlorite. To evaluate the physiological function of the ttcA in P. aeruginosa PAO1 against various stresses, a gene deletion mutant
(ΔttcA) was constructed in PAO1, as described in the Methods. Resistance levels against stresses including: osmotic (high salt, 5 mM NaCl), heat (high temperature, 50 °C), acidic (pH 4), basic (alkaline, pH 9) and oxidative stresses, were determined using a plate sensitivity assay and were compared to that of wild-type PAO1. The oxidants used in this study included: H₂O₂, sodium hypochlorite (NaOCl), organic hydroperoxides (cumene hydroperoxide [CHP]), superoxide generators (paraquat [PQ]), a thiol-depleting agent (N-ethylmaleimide [NEM]) and an intracellular iron chelating agent 2,2’-dipyridyl (DIPY). There were no significant differences in the resistance levels of the ΔttcA mutant and wild-type PAO1 against high salt, high temperature, acidic pH, basic pH, organic hydroperoxides, superoxide generators, the thiol-depleting agent and the iron chelator (Fig. 3a). However, the ΔttcA mutant exhibited 50-fold lower resistance to H₂O₂ and an 8-fold reduction in the percent survival against NaOCl compared to PAO1 (Fig. 3a). The sensitive phenotype of the ΔttcA mutant against both H₂O₂ and NaOCl was complemented by the expression of a single copy of ttcA in Tn7 site (Fig. 3a), indicating that TtcA plays an important role in the H₂O₂-mediated and NaOCl-derived stress response. In PAO1, the cellular detoxification of H₂O₂ primarily depended on catalase activity levels; however, other mechanisms, such as thiol-peroxidase activity (Tpx) and supporting systems, including haem biosynthesis, were also required to achieve fully responsive functionality against H₂O₂ in P. aeruginosa. NaOCl is a bleaching agent that acts as a strong oxidizing agent and can disturb several enzymatic mechanisms, both redox and non-redox, including reactions in tRNA modification processes. Moreover, NaOCl has been shown to generate intracellular ROS, which may increase H₂O₂ levels and lead to mutant susceptibility. Although our results showed that P. aeruginosa TtcA particularly affects the oxidative stress response, it may also alter the translational process of other biological pathways since it could function in the translational control.

Furthermore, PAO1 containing an extra copy of functional ttcA did not elevate the levels of resistance against these tested oxidants (Fig. 3a), suggesting that other components in the tRNA modification process are required or another detoxification system compensates for oxidant sensitivities. In addition to TtcA in P. aeruginosa PAO1, TrmJ, another tRNA-modifying enzyme, has also been shown to function in the oxidative stress response of P. aeruginosa PA14.

[Fe-S] cluster-ligated cysteine residues are required for the physiological function of TtcA. To assess the important role of [Fe-S] clusters in TtcA-mediated protection against stress conditions, the site-directed mutagenesis of ttcA and a complementation assay were performed. Amino acids were changed from cysteine

(ΔttcA) was constructed in PAO1, as described in the Methods. Resistance levels against stresses including: osmotic (high salt, 5 mM NaCl), heat (high temperature, 50 °C), acidic (pH 4), basic (alkaline, pH 9) and oxidative stresses, were determined using a plate sensitivity assay and were compared to that of wild-type PAO1. The oxidants used in this study included: H₂O₂, sodium hypochlorite (NaOCl), organic hydroperoxides (cumene hydroperoxide [CHP]), superoxide generators (paraquat [PQ]), a thiol-depleting agent (N-ethylmaleimide [NEM]) and an intracellular iron chelating agent 2,2’-dipyridyl (DIPY). There were no significant differences in the resistance levels of the ΔttcA mutant and wild-type PAO1 against high salt, high temperature, acidic pH, basic pH, organic hydroperoxides, superoxide generators, the thiol-depleting agent and the iron chelator (Fig. 3a). However, the ΔttcA mutant exhibited 50-fold lower resistance to H₂O₂ and an 8-fold reduction in the percent survival against NaOCl compared to PAO1 (Fig. 3a). The sensitive phenotype of the ΔttcA mutant against both H₂O₂ and NaOCl was complemented by the expression of a single copy of ttcA in Tn7 site (Fig. 3a), indicating that TtcA plays an important role in the H₂O₂-mediated and NaOCl-derived stress response. In PAO1, the cellular detoxification of H₂O₂ primarily depended on catalase activity levels; however, other mechanisms, such as thiol-peroxidase activity (Tpx) and supporting systems, including haem biosynthesis, were also required to achieve fully responsive functionality against H₂O₂ in P. aeruginosa. NaOCl is a bleaching agent that acts as a strong oxidizing agent and can disturb several enzymatic mechanisms, both redox and non-redox, including reactions in tRNA modification processes. Moreover, NaOCl has been shown to generate intracellular ROS, which may increase H₂O₂ levels and lead to mutant susceptibility. Although our results showed that P. aeruginosa TtcA particularly affects the oxidative stress response, it may also alter the translational process of other biological pathways since it could function in the translational control.

Furthermore, PAO1 containing an extra copy of functional ttcA did not elevate the levels of resistance against these tested oxidants (Fig. 3a), suggesting that other components in the tRNA modification process are required or another detoxification system compensates for oxidant sensitivities. In addition to TtcA in P. aeruginosa PAO1, TrmJ, another tRNA-modifying enzyme, has also been shown to function in the oxidative stress response of P. aeruginosa PA14.

[Fe-S] cluster-ligated cysteine residues are required for the physiological function of TtcA. To assess the important role of [Fe-S] clusters in TtcA-mediated protection against stress conditions, the site-directed mutagenesis of ttcA and a complementation assay were performed. Amino acids were changed from cysteine

(ΔttcA) was constructed in PAO1, as described in the Methods. Resistance levels against stresses including: osmotic (high salt, 5 mM NaCl), heat (high temperature, 50 °C), acidic (pH 4), basic (alkaline, pH 9) and oxidative stresses, were determined using a plate sensitivity assay and were compared to that of wild-type PAO1. The oxidants used in this study included: H₂O₂, sodium hypochlorite (NaOCl), organic hydroperoxides (cumene hydroperoxide [CHP]), superoxide generators (paraquat [PQ]), a thiol-depleting agent (N-ethylmaleimide [NEM]) and an intracellular iron chelating agent 2,2’-dipyridyl (DIPY). There were no significant differences in the resistance levels of the ΔttcA mutant and wild-type PAO1 against high salt, high temperature, acidic pH, basic pH, organic hydroperoxides, superoxide generators, the thiol-depleting agent and the iron chelator (Fig. 3a). However, the ΔttcA mutant exhibited 50-fold lower resistance to H₂O₂ and an 8-fold reduction in the percent survival against NaOCl compared to PAO1 (Fig. 3a). The sensitive phenotype of the ΔttcA mutant against both H₂O₂ and NaOCl was complemented by the expression of a single copy of ttcA in Tn7 site (Fig. 3a), indicating that TtcA plays an important role in the H₂O₂-mediated and NaOCl-derived stress response. In PAO1, the cellular detoxification of H₂O₂ primarily depended on catalase activity levels; however, other mechanisms, such as thiol-peroxidase activity (Tpx) and supporting systems, including haem biosynthesis, were also required to achieve fully responsive functionality against H₂O₂ in P. aeruginosa. NaOCl is a bleaching agent that acts as a strong oxidizing agent and can disturb several enzymatic mechanisms, both redox and non-redox, including reactions in tRNA modification processes. Moreover, NaOCl has been shown to generate intracellular ROS, which may increase H₂O₂ levels and lead to mutant susceptibility. Although our results showed that P. aeruginosa TtcA particularly affects the oxidative stress response, it may also alter the translational process of other biological pathways since it could function in the translational control.

Furthermore, PAO1 containing an extra copy of functional ttcA did not elevate the levels of resistance against these tested oxidants (Fig. 3a), suggesting that other components in the tRNA modification process are required or another detoxification system compensates for oxidant sensitivities. In addition to TtcA in P. aeruginosa PAO1, TrmJ, another tRNA-modifying enzyme, has also been shown to function in the oxidative stress response of P. aeruginosa PA14.

[Fe-S] cluster-ligated cysteine residues are required for the physiological function of TtcA. To assess the important role of [Fe-S] clusters in TtcA-mediated protection against stress conditions, the site-directed mutagenesis of ttcA and a complementation assay were performed. Amino acids were changed from cysteine
(C) to serine (S) at different positions in the TtcA, including a cysteine next to the PP-loop motif C38; putative cysteines for [Fe-S] cluster ligation at C115, C118 and C206; and other conserved cysteines, C184 and C203, using pUC18-mini-Tn7T-Gm-\(ttcA\); then, mutated genes were transformed and integrated into the chromosome of the \(\Delta ttcA\) mutant. A plate sensitivity assay using lethal concentrations of H\(_2\)O\(_2\) and NaOCl was performed to compare the susceptibility of bacterial growth between the transformed \(\Delta ttcA\) mutants. The results in Fig. 3b show that increased susceptibility to H\(_2\)O\(_2\) in the \(\Delta ttcA\) mutant was completely restored to wild-type PAO1 levels in \(\Delta ttcA\) mutants containing either the native \(ttcA\) cassette (WT), the site-directed \(ttcA\) cassette with C38S, or C184S. However, H\(_2\)O\(_2\) susceptibility in the \(\Delta ttcA\) mutant containing the site-directed \(ttcA\) cassette with either C115S, C118S, C203S or C206S demonstrated similar levels as the \(\Delta ttcA\) mutant (Fig. 3b), indicating no phenotypic restoration among these site-directed mutant strains. Therefore, the four cysteines (C115, C118, C203 and C206) were required for fully functional TtcA to play a role in the H\(_2\)O\(_2\)-mediated stress response. Moreover, a similar pattern was obtained with the NaOCl complementation assay, as shown in Fig. 3c, indicating that the site-directed \(ttcA\) cassette containing the cysteine residues (either C115S, C118S, C203S or C206S) was unable to restore NaOCl susceptibility of the \(\Delta ttcA\) mutant to wild-type PAO1 levels, resulting in a sensitivity level similar to the \(\Delta ttcA\) mutant. This suggested that these four cysteines were also required for the TtcA functionality in the NaOCl-mediated stress response. Similar observations regarding the importance of this conserved Cys-X-X-Cys motif in the TtcA protein have been reported for the thiolation of the cytidine in position 32 of tRNA in S. Typhimurium\(^{19}\) and in E. coli\(^{28}\). Mutation of C219 to alanine in E. coli TtcA, mapped as C203 of P. aeruginosa TtcA, exhibited a 50% reduction in tRNA thiolation activity\(^{19}\), which was supported by the observed importance of this cysteine residue for the full function of TtcA. However, the exact function of these four cysteine residues in the TtcA, i.e. their possible involvement in the [Fe-S] cluster ligation, is under investigation.

The \(\Delta ttcA\) mutant exhibits decreased total catalase activity via KatA function. In several pathogenic bacteria, the cellular detoxification of H\(_2\)O\(_2\) mainly depends on catalase activity levels. The two major catalases KatA and KatB are responsible for cellular H\(_2\)O\(_2\) detoxification in P. aeruginosa PAO1\(^{11,30}\). To investigate the involvement of TtcA in the H\(_2\)O\(_2\)-mediated stress response through catalase activity, a total intracellular catalase activity assay was performed in wild-type PAO1 and the \(\Delta ttcA\) mutants. The results showed that total catalase activity in the \(\Delta ttcA\) mutant was 39% and 41% lower than that in wild-type PAO1 under the exponential
and stationary phases, respectively, while the \( \Delta \)ttcA mutant harbouring a functional ttcA cassette at the Tn7 site showed catalase activity levels similar to that of wild type (Fig. 4a). This result suggested that TtcA is required for full catalase activity in \( P. \) aeruginosa \( PAO1 \) under both the exponential and stationary phases.

To determine whether TtcA has roles in KatA or KatB activity, a catalase gel activity assay was performed. The results in Fig. 4b show that KatA activity in the \( \Delta \)ttcA mutant was decreased compared to that in wild-type \( PAO1 \), and activity was restored by the expression of the functional ttcA cassette at the Tn7 site, as shown in the complemented strain. However, KatB activity levels were slightly increased in the \( \Delta \)ttcA mutant compared to those in wild-type \( PAO1 \) (Fig. 4b). These data support the hypothesis that TtcA might have a direct role in KatA activity and may disrupted the H\(_2\)O\(_2\)-responsive pathways. To confirm the contribution of TtcA to KatA activity, double \( \Delta \)katA \( \Delta \)ttcA and \( \Delta \)katB \( \Delta \)ttcA mutants were constructed and used to determine H\(_2\)O\(_2\) susceptibility levels in a plate sensitivity assay. If TtcA plays a role in KatA function, the sensitivity of the double mutant should be the same as that of the \( \Delta \)katA mutant. By contrast, if TtcA plays no role in the maintenance of KatA activity, the sensitivity in the double mutant should be additive compared to the single \( \Delta \)katA mutant. The results in Fig. 4c show that the \( \Delta \)katA mutant was more than 1,000-fold more sensitive to H\(_2\)O\(_2\), while the \( \Delta \)ttcA mutant was approximately 50-fold less sensitive to H\(_2\)O\(_2\) compared to the sensitivity of wild-type \( PAO1 \) (Fig. 4b). These data support the hypothesis that TtcA might have a direct role in KatA activity and disrupted the H\(_2\)O\(_2\)-responsive pathways. To confirm the contribution of TtcA to KatA activity, double \( \Delta \)katA \( \Delta \)ttcA and \( \Delta \)katB \( \Delta \)ttcA mutants were constructed and used to determine H\(_2\)O\(_2\) susceptibility levels in a plate sensitivity assay. If TtcA plays a role in KatA function, the sensitivity of the double mutant should be the same as that of the \( \Delta \)katA mutant. By contrast, if TtcA plays no role in the maintenance of KatA activity, the sensitivity in the double mutant should be additive compared to the single \( \Delta \)katA mutant. The results in Fig. 4c show that the \( \Delta \)katA mutant was more than 1,000-fold more sensitive to H\(_2\)O\(_2\), while the \( \Delta \)ttcA mutant was approximately 50-fold less sensitive to H\(_2\)O\(_2\) compared to the sensitivity of wild-type \( PAO1 \). Additionally, the double \( \Delta \)katA \( \Delta \)ttcA mutant exhibited H\(_2\)O\(_2\) susceptibility levels similar to the \( \Delta \)katA mutant under a range of H\(_2\)O\(_2\) concentrations with differing lethality (Fig. 4c), suggesting that TtcA contributes to KatA activity against H\(_2\)O\(_2\) toxicity. Moreover, the results in Fig. 4d show that the \( \Delta \)katB\(^{-}\) mutant was approximately 100-fold more sensitive to H\(_2\)O\(_2\), while the double \( \Delta \)katB\(^{-}\) \( \Delta \)ttcA mutant exhibited increased H\(_2\)O\(_2\) susceptibility (20-fold) relative to the \( \Delta \)katB\(^{-}\) mutant (Fig. 4d), suggesting that TtcA contributes to H\(_2\)O\(_2\) resistance primarily through regulation of KatA activity.

Deletion of ttcA causes a change in the expression of genes involved in the oxidative stress response. To test whether the deletion of ttcA contributed to decreased KatA activity either at the transcriptional level or at the post-transcriptional level, expression analysis of katA in the \( \Delta \)ttcA mutant compared to wild-type \( PAO1 \) was performed using real time RT-PCR analysis. The results in Fig. 5a show that katA expression in the \( \Delta \)ttcA mutant was approximately three-fold higher than that in \( PAO1 \) under conditions lacking an oxidant,
and increased katA expression in the ΔttcA mutant was fully restored to wild-type levels by the extra copy of functional ttcA inserted at the Tn7 site. This suggests that decreased KatA activity in the ΔttcA mutant does not result from altered katA expression at the transcriptional level; however, it may arise from post-transcriptional control, as it has previously been shown that TtcA has roles in translational control and decreased KatA activity was observed in this study. To extend our gene expression analysis, the expression profile of genes involved in the oxidative stress response, such as katB, oxyR, and tpx, was determined using plate sensitivity assays and are shown as the mean and SD of the percent survival from three independent experiments.

**Figure 4.** Catalase activity in *P. aeruginosa* strains. (a) Total intracellular catalase activity was determined in both exponential and stationary cultures of *P. aeruginosa* strains. The data shown are the mean and SD of catalase specific activities in each strain from three independent experiments. The asterisk indicates statistical significance (*p* < 0.05) compared with PAO1::Tn at the same growth phase. (b) KatA and KatB catalase gel activities were investigated among *P. aeruginosa* strains. The band intensity of each tested strain was calculated as the relative intensity (fold change) compared to that of PAO1::Tn, with an asterisk indicating statistical significance (*p* < 0.05). The full-length gel was shown in the Supplementary Fig. S1. H2O2 resistance levels in the double ΔkatAΔttcA (c) and katB−ΔttcA (d) mutants compared to that of the PAO1 wild type and the single ΔttcA, ΔkatA, and katB− mutants were determined using plate sensitivity assays and are shown as the mean and SD of the percent survival from three independent experiments.
NaOCl treatment (see Supplementary Fig. S3). All changes in gene expression in the ΔttcA mutant were restored to wild-type levels by a chromosomal insertion of the extra copy of ttcA. This suggested that the ΔttcA mutant caused a defect in H₂O₂ detoxification via KatA-mediated mechanisms, leading to a global change in gene expression, including katB, oxyR and tpx expression, in response to H₂O₂-mediated oxidative stress. This result supports the previous observation in Fig. 4b that KatB activity was slightly increased in the ΔttcA mutant compared to the activity in wild-type PAO1. However, the real time RT-PCR analysis could reflect an effect on both transcriptional alteration and the stability of mRNAs.

Furthermore, to observe the translational efficiency of the katA transcript, Western blot analysis was performed using an ectopic 6His-tagged katA expression vector on the ΔkatA mutant background to compare the native ttcA (ΔkatA) and ttcA deletion (ΔkatAΔttcA) strains. The results shown in Fig. 5b indicate that the relative amounts of 6His-KatA in the ttcA deletion mutant (ΔkatAΔttcA/pkatA-6His, 36%) were dramatically lower than those in the native ttcA mutant (ΔkatA/pkatA-6His, 100%) and were partially restored by the extra copy of ttcA under Tn7-mediated expression (ΔkatAΔttcA/pkatA-6His, 67%). These results suggested a defect in the translational efficiency of 6His-tagged katA expression in the absence of functional ttcA and indicated that ttcA plays roles in the oxidative stress response at the post-transcriptional level via KatA activity, and the disruption of functional ttcA alters the global expression profile of genes involved in oxidative stress management, including induction of katB and expression of tpx. Western analysis showed only the steady state level of KatA in the cell and therefore could not exclude an effect on KatA degradation.

The ΔttcA mutant shows attenuated virulence in a Drosophila host model. The full function of KatA is required for bacterial virulence in several model host systems, as shown in previous studies, and TtcA has been shown to respond to oxidative stress via KatA activity; therefore, the contribution of ttcA to the bacterial pathogenicity of P. aeruginosa was evaluated using Drosophila melanogaster as a pathogen–host model. As shown in Fig. 6a, feeding the flies with cultured PAO1 resulted in 50.8 ± 12.5% (after incubation for 15 hours) and 36.0 ± 6.7% (after incubation for 24 hours) fly survival compared with 100 ± 0% (at both time points) fly survival when LB medium was fed to the flies as a negative control. Feeding the flies with ΔttcA mutants resulted in 1.6-fold and 2.1-fold increases in fly survival (81.7 ± 7.4% and 76.7 ± 7.2% after incubation for 15 and 24 hours, respectively) compared with feeding with PAO1 (Fig. 6a). Thus, ttcA deletion attenuated the virulence of P. aeruginosa PAO1 in the tested model (p < 0.01). The attenuated virulence phenotype of the ΔttcA mutant was restored in a ΔttcA mutant expressing a functional copy of ttcA (57.2 ± 8.2% and 40.5 ± 6.3% fly survival after incubation for 15 and 24 hours, respectively). The attenuated virulence phenotype was consistent with H₂O₂ sensitivity levels in the ΔttcA mutant (Fig. 3a). In several plant and animal pathogenic bacteria, defects in peroxide detoxification or repair systems, such as knockout of catalase, methionine sulfoxide reductase or iron-sulfur cluster regulator
genes, render the mutant strains attenuated for virulence in model hosts. Hydrogen peroxide is one of the key components of innate immunity generated by host cells to eradicate invading microbes. In human hosts, H₂O₂ is produced within the phagolysosomes of phagocytic cells to kill engulfed pathogens. Thus, defective protection against H₂O₂ toxicity in bacteria would reduce survival within the host. Hence, the attenuated phenotype may result from the reduced ability of the ΔttcA mutant to mitigate exposure to H₂O₂ during host interactions.

Moreover, the four cysteine residues in TtcA were required for fully functionality in the oxidative stress response via catalase activity. To investigate the requirement of these TtcA cysteine residues in bacterial virulence, complementation with the site-directed ΔttcA mutants was evaluated in a Drosophila feeding assay. The results shown in Fig. 6b are similarly to those in Fig. 6a, indicating that feeding the flies with cultured PAO1 either with or without Tn7-mediated insertion of a ΔttcA expression cassette and incubation for 18 hours resulted in approximately 50% fly survival; however, feeding with ΔttcA mutants resulted in an approximately two-fold increase in fly survival. Feeding with the ΔttcA::Tn-TtcA mutant resulted in a fly survival level similar to that of the PAO1 strain (Fig. 6b). Substitution of these particular cysteine residues, either C115, C118, C203, or C206, with serine in the functional ΔttcA expression cassette and insertion into the ΔttcA mutant chromosome did not restore fly survival levels, while replacing one of the other conserved cysteines (either C38 or C184) in TtcA caused the phenotypic restoration of fly survival to wild-type PAO1 levels (Fig. 6b). This indicated that these four particular cysteine residues, including putative amino acids for iron-sulfur cluster ligation (C115, C118 and C206), were required for the complete functionality of TtcA in bacterial pathogenicity. Several P. aeruginosa genes involved in iron-sulfur cluster biogenesis, including IscR, have been shown to play a role in bacterial virulence, which may correlate with TtcA function in the H₂O₂-mediated oxidative stress response through catalase activity.

**ttcA expression is increased in response to H₂O₂ and NaOCl exposure.** Adaptive gene expression is a key component of bacterial defence against environmental stresses. The expression of many genes involved in oxidative stress protection and repair processes is frequently induced by exposure to oxidants. The expression patterns of ttcA in PAO1 cultivated under inducing concentrations of various oxidants were determined using real time RT-PCR. The results illustrated that exposure of PAO1 to organic hydroperoxides, superoxide anion-generating agents, a thiol-chelating agent and an iron-chelating agent did not induce ttcA expression (Fig. 7a). By contrast, H₂O₂ and NaOCl treatment of PAO1 highly induced ttcA expression by 13.4 ± 1.5-fold and 2.9 ± 1.1-fold, respectively (Fig. 7a). The induction of ttcA expression by H₂O₂ and NaOCl treatment correlated with physiological analysis indicating that TtcA contributes to protection against H₂O₂ and NaOCl.
To extend the range of the oxidant-induced gene expression profile, several concentrations of oxidants were applied to bacterial cultures and analysed by real time RT-PCR. The results in Fig. 7b showed that PAO1 cultures were induced with H₂O₂ at concentrations ranging from 0.2 mM to 1 mM and in a dose-dependent manner, which was similar to the gene expression profile obtained for genes in the OxyR regulon, including katA, katB, ahpB and ahpCF. This hinted at the possibility that ttcA expression is regulated by OxyR, the global transcriptional regulator responding to H₂O₂. Moreover, extending the concentration range for NaOCl treatment from 0.001% to 0.02% showed that ttcA expression was not significantly altered under 0.005% NaOCl exposure compared to that in untreated PAO1 (Fig. 7c). This suggested that, unlike H₂O₂ induction, induction in response to high concentrations of NaOCl (including a sublethal dose of 0.02%), which slightly induced ttcA expression (2.5-fold), may arise from NaOCl reactions generating oxidative stress and probably did not arise via direct NaOCl reactions with the regulator. The NaOCl induction mechanism is under further investigation.

OxyR modulates the expression of ttcA to control catalase activity under stress exposure. ttcA promoter analysis was performed and physically mapped in silico, and the results are presented in Fig. 7d. ttcA is located next to PA1191 with a 102-bp intergenic region. To characterize the ttcA promoter, putative +1 sites were investigated using 5′ RACE. The +1 site of ttcA was mapped to a cytosine located 28 bp upstream of its translational ATG start codon (Fig. 7d). Two sequences (GGGCTG and GCGTAAAAT, separated by 18 bp) that resembled the E. coli -70 and -10 promoter motifs were identified. Given the limited intergenic space and a putative promoter sequence analysis, the ttcA and PA1191 promoter motifs might overlap with each other. The canonical OxyR promoter recognition sequence was previously proposed to be ATAG-N7-CTAT-N7-ATAG-N7-CTAT. We mapped the P. aeruginosa ttcA promoter region and found an upstream sequence (TCGGctcgtTTGctgtaaaATAGccagctTTCT) that matched 56% (9 of 16 bases) of the OxyR promoter recognition sequence; therefore, we considered this sequence a putative OxyR binding domain of ttcA (Fig. 7d).
putative binding domain overlapped the -10 promoter region, implying an OxyR derepression mechanism for \( \text{ttcA} \) expression in response to oxidative stress. OxyR is a member of the LysR family of transcription regulators, which often use extended palindromic DNA sequences as binding boxes to modulate target gene expression, and diverse consensus sequences for OxyR binding boxes in target gene promoters have been proposed. Direct binding of OxyR to the \( \text{ttcA} \) promoter was determined by electrophoresis mobility shift assay (EMSA). The results in the Fig. 8a demonstrated that purified OxyR binds specifically to the putative \( \text{ttcA} \) promoter in the presence of the reducing agent, dithiothreitol (DTT), suggesting that reduced OxyR is required for the \( \text{ttcA} \) promoter binding. These results support the hypothesis that OxyR directly binds to the \( \text{ttcA} \) promoter and may regulate \( \text{ttcA} \) expression.

To assess whether OxyR regulated the induction of \( \text{ttcA} \) expression upon exposure to oxidative stress, \( \text{ttcA} \) expression levels were examined in an \( \Delta \text{oxyR} \) mutant (\( \Delta \text{oxyR}/\text{pBBR} \)) and the complemented mutant (\( \Delta \text{oxyR}/\text{pOxyR} \)) grown under uninduced, 0.5 mM \( \text{H}_2\text{O}_2 \), or 0.02% NaOCl induced conditions were investigated using real time RT-PCR and analysed as described in previous experiments. The asterisks indicate statistically significant differences (\( p < 0.01 \)) compared with uninduced conditions. (c) Proposed model of OxyR-regulated \( \text{katA} \) and \( \text{ttcA} \) expression under oxidative stress conditions. \( \text{P. aeruginosa} \) OxyR upregulates \( \text{katA} \) and \( \text{ttcA} \) expression to increase catalase activities in response to \( \text{H}_2\text{O}_2 \) generated by host defence mechanisms. The four cysteine residues required for fully functional TtcA activity to have a role in the oxidative stress response via KatA activity and facilitates bacterial survival during infection.

**Figure 8.** OxyR directly regulates \( \text{ttcA} \) expression. (a) Electrophoretic mobility shift assay was performed using \( ^{32}\text{P} \)-labeled \( \text{ttcA} \) promoter fragment and increasing concentrations of purified OxyR in the presence of 100 mM DTT. F and B indicate free and bound probes, respectively. The results from three replications and the full-length gel images were shown in the Supplementary Fig. S4. (b) Expression levels of \( \text{ttcA} \) in wild-type \( \text{PAO1} \) (\( \text{PAO1}/\text{pBBR} \)), the \( \Delta \text{oxyR} \) mutant (\( \Delta \text{oxyR}/\text{pBBR} \)) and the complemented mutant (\( \Delta \text{oxyR}/\text{pOxyR} \)) grown under uninduced, 0.5 mM \( \text{H}_2\text{O}_2 \), or 0.02% NaOCl induced conditions were investigated using real time RT-PCR and analysed as described in previous experiments. The asterisks indicate statistically significant differences (\( p < 0.05 \)) compared with uninduced conditions. (c) Proposed model of OxyR-regulated \( \text{katA} \) and \( \text{ttcA} \) expression under oxidative stress conditions. \( \text{P. aeruginosa} \) OxyR upregulates \( \text{katA} \) and \( \text{ttcA} \) expression to increase catalase activities in response to \( \text{H}_2\text{O}_2 \) generated by host defence mechanisms. The four cysteine residues required for fully functional TtcA activity to have a role in the oxidative stress response via KatA activity and facilitates bacterial survival during infection.
Exponential phase cells (OD 600 of 0.5) were used in all experiments. All plasmids used in this study are listed in Supplementary Table S1. Strains were aerobically cultivated in Lysogeny broth (LB from BD Difco, USA) at 37 °C unless otherwise stated. pBBR-TtcA using primers BT4675 and BT4676. GE Healthcare. A 247-bp fragment of the ttcA coding region used as a gene-specific probe was amplified from P. aeruginosa strains were carried out using electroporation as previously described. The oligonucleotide primers used in this study are listed in Supplementary Table S2.

Construction of P. aeruginosa ΔttcA mutants. The ttcA deletion mutant was constructed using homologous recombination with an unmarked Cre-loxP system as previously described. A 1,271-bp right-flank (RF) containing the C-terminal of the ttcA coding region and a 1,093-bp left-flank (LF) containing the N-terminal was separately amplified from PAO1 genomic DNA using primers EBI1009 and EBI1010 and primers EBI1007 and EBI1008, respectively. The RF fragment was digested with PstI and the 1,010-bp RF fragment was isolated and ligated into pUC18::Gmr digested with HindIII/blunted and PstI yielding pUCΔttcA::Gmr. The LF fragment was digested with NcoI and the 931-bp LF fragment was isolated and ligated into pUC18::Gmr digested with XhoI/blunted and PstI yielding pUCΔttcA::Gmr. The constructed plasmid resulted in the deletion of 721 bp of the ttcA coding region and a 1,093-bp left-flank containing the N-terminal was amplified from PAO1 genomic DNA using primers EBI1009 and EBI1010 and primers EBI1007 and EBI1008, respectively. The RF fragment was digested with PstI and the 1,010-bp RF fragment was isolated and ligated into pUC18::Gmr digested with HindIII/blunted and PstI yielding pUCΔttcA::Gmr. The constructed plasmid resulted in the deletion of 721 bp of the ttcA coding region. pUCΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr and ΔttcA::Gmr was transferred into PAO1, and the ΔttcA::Gmr mutants were selected for the Gmr.

Construction of plasmid and mini-Tn7 harbouring ttcA-coding regions. A pBBR-TtcA for ectopic expression of ttcA was constructed by amplifying the full-length ttcA with primers BT4673 and BT4674. The 688-bp PCR products were cloned into the medium-copy-number expression vector pBBR1MCS-4 cut with SmaI, yielding pBBR-TtcA. Single-copy complementation was performed using a mini-Tn7 system. The full-length ttcA were cut from pBBR-TtcA and cloned into pUC18-mini-Tn7::Gm-LAC prior to transposing into either PAO1 or mutant strains, generating overexpressed (PAO1::Tn-ttcA) or complemented (ΔttcA::Tn-ttcA) strains.

Construction of ΔoxyR mutant and plasmid harbouring oxyR-coding regions. The oxyR deletion mutant was constructed as similar as the ttcA deletion mutant construction excepting with primers, BT5910 and BT5911, and a 625-bp deletion site in the oxyR-coding region was in between restriction enzymes, XhoI/blunted and SacII. A pBBR-OxyR for ectopic expression of oxyR was constructed as similar as pBBR-TtcA construction excepting with primers, EBI1047 and EBI1048.
Construction of katB\(^{-}\) and ΔttcAkatB\(^{-}\) mutants. The katB knockout mutant was constructed by an insertional inactivation method using pKNOCK vector\(^{44}\) as previously described\(^{35}\). The katB fragment amplified from PA01 genomic DNA with primers, BT5639 and BT5640, was cloned into pKNOCK\(_{gal}\), digested with SmaI, generating pKNOCK\(_{gal}\)-katB, which was introduced into PA01 by conjugation. The trans-conjugants were selected by the Gm\(^{+}\) phenotype. To construct a double ΔttcAkatB\(^{-}\) mutant, the pKNOCK\(_{gal}\)-katB was introduced into the genome of ΔttcA mutant. The mutants were confirmed by PCR and DNA sequencing.

Site-directed mutagenesis of TtcA. Site-directed mutagenesis was performed to convert cysteine residues (C38, C115, C118, C184, C203, or C206) to serine residues through PCR-based mutagenesis as previously described\(^{34}\). To construct pTn-\(-\)ttcA38S for the expression of TtcA-C38S, two pairs of primers EBI1011-TN7S and EBI1012-BT5250, were used in two-step PCR using pUC18-mini-Tn7-Gm-\(-\)ttcA as a template. The PCR product was digested with EcoRI and Sacl prior to cloning into pUC18-mini-Tn7-Gm-LAC, generating pTn-\(-\)ttcA38S. pTn-\(-\)ttcA115S, pTn-\(-\)ttcA118S, pTn-\(-\)ttcA184S, pTn-\(-\)ttcA203S and pTn-\(-\)ttcA206S were constructed using the same protocol with different sets of mutagenic primers: EBI1013 and EBI1014 for C115S, EBI1015 and EBI1016 for C118S, EBI1017 and EBI1018 for C184S, EBI1019 and EBI1020 for C203S, and EBI1021 and EBI1022 for C206S. The presence of each mutation was verified by DNA sequencing.

Expression and purification of P. aeruginosa TtcA. 6His-tagged TtcA from P. aeruginosa was expressed using the pQE-30Xa expression system (Qiagen, Germany) under oxygen-limited conditions as previously described\(^{34}\). The full-length ttcA gene was amplified from PA01 genomic DNA with the primers EBI1035 and EBI1036. An 835-bp PCR product was digested with HindIII prior to ligation into pQE-30Xa digested with StuI/blunted and HindIII to generate pQE-30Xa-\(-\)ttcA for the high-level expression of ttcA containing a N-terminal 6His-tag. An E. coli M15 strain harbouring pQE-30Xa-\(-\)ttcA was grown to an OD\(_{600}\) of 1.0 before being induced with 100 mM IPTG for 60 min with 40 rpm shaking. Purification of 6His-tagged TtcA was carried out using a nickel-nitriilotriacetic acid (Ni-NTA) agarose column as previously described\(^{34}\). The purity of the TtcA protein was more than 95%, as judged by a major band corresponding to the 32.3-kDa protein observed on SDS-PAGE. UV-Visible spectrophotometry was used to analyse the relative amount of [Fe-S] cluster using the ratio of absorbance 420 nm and 280 nm.

Plate sensitivity assay. A plate sensitivity assay was performed to determine the oxidant resistance level as previously described\(^{3}\). Briefly, exponential-phase cells were adjusted to OD\(_{600}\) of 0.1 before making 10-fold serial dilutions. 10 µl of each dilution was then spotted onto LB agar plate containing testing reagents. The plates were incubated overnight at 37 °C before the colony forming units (CFU) were scored. Percent survival was defined as the percentage of the CFU on plates containing oxidant divided by the CFU on plates without oxidant.

Hydrogen peroxide, NaOCl, heat and pH susceptibility test. A susceptibility assay was performed to determine the stress resistance level as previously described\(^{35}\). In short, exponential-phase cultures were normalized to an OD\(_{600}\) of 0.1 before treating with lethal concentrations of either H\(_2\)O\(_2\), NaOCl, heat, acidic pH, or basic pH for 30 min at 37 °C. After treatment, cells were immediately washed twice with fresh LB broth. Cells that survived the treatment were scored using a viable cell count. The resistance levels against these stresses were expressed as the % survival, defined as the percentage of the CFU with treatment divided by the CFU without treatment.

Catalase activity assays. Total catalase activity in P. aeruginosa cells was measured by spectrophotometrically monitoring the decomposition of hydrogen peroxide\(^{3}\). Briefly, the reaction was performed by mixing bacterial lysate with 30 mM H\(_2\)O\(_2\) in 50 mM phosphate buffer pH 7.0. The absorbance changes at A\(_{240}\) were recorded at time intervals and calculated as the specific activity of catalase (U mg\(^{-1}\) protein). One unit of catalase was defined as the amount of enzyme required to hydrolyse 1 µmol of H\(_2\)O\(_2\) per min at 25 °C, pH 7.0, and the molar extinction e\(_{240}\) was equal to 0.041 cm\(^{-1}\) µmol\(^{-1}\).

The gel activity of Kat was intensely measured from native PAGE of P. aeruginosa cell extracts, which were stained for Kat activity as previously described\(^{45}\). Thirty milligrams (unheated) of protein were loaded, and protein concentrations were estimated using Bradford assay (Bio-Rad, USA). The stained gel was renatured, and catalase activity was visualized following a previously described method\(^{46}\) with some modifications by washing twice before soaking with horseradish peroxidase (Sigma, USA) and then removing this enzyme. The gel was immediately soaked in 5 mM H\(_2\)O\(_2\) and stained with 3,3′-diaminobenzidine. Catalase activity was visualized as colourless bands against a brownish background.

Western blot analysis. Western blot analysis was performed as previously described\(^{47}\). In brief, crude protein was extracted and isolated before mixing with 6X protein loading dye and boiling for 10 min. The stained protein was run under 12.5% SDS-PAGE and transferred to a Hybond PVDF membrane (GE Healthcare) in a semi-dry transfer cell (Bio-Rad). The transferred membrane was blocked and hybridized with anti-6His-peroxidase primary antibody (Roche, Switzerland) and developed with Ultra TMB-Blotting Solution (Thermo Scientific) according to the manufacturer’s recommendation.

Drosophila virulence test. The virulence of P. aeruginosa was investigated using the Drosophila melanogaster feeding assay as previously described\(^{3}\). Shortly, 800 µL of P. aeruginosa cultures were overlaid to completely cover the surface of the corn flour Drosophila medium in a glass fly culture vial. One-week-old adult flies were starved for 3 hours prior to the feeding assay. Twenty flies were added to each vial and incubated at 25 °C before the number of the viable flies was observed at different time points. The experiments were performed in a double-blind fashion and were analyzed from nine experiments using three different batches of flies.
Real time RT-PCR. RNA extraction and reverse transcription was performed as previously mentioned.6,35 Real time RT-PCR was conducted using a SYBR® FAST qPCR kit (KAPA Biosystems, USA). The reaction was run on an Applied Biosystems StepOnePlus thermal cycler under the recommended fast protocol condition. The specific primer pairs used for ttcA, katA, katB, oxyR, and tpx were BT4765–BT4766, BT5637–BT5638, BT5639–BT5640, EBI163–EBI164 and BT3186–BT3187, respectively. The primer pair for the 16S rRNA gene was BT2781–BT2782, which was used as the normalizing gene. Relative expression analysis was calculated using StepOne software and is presented as expression fold-change relative to the level of uninduced conditions. Data shown are the means with standard deviations (SD) from three biologically independent experiments.

5′ rapid amplification of cDNA ends (RACE). 5′ RACE was performed using a 5′/3′ RACE kit (Roche, Switzerland) as previously described.19 Essentially, DNase I-treated total RNA was reverse transcribed using specific primers EBI341 as SP1 primers. The first-strand DNA (cDNA) was purified, and poly(A) was added to the 5′-terminus of the cDNA using terminal transferase. Next, poly(A)-tailed cDNA was PCR-amplified using the specific SP2 primer BT4991 and an anchored oligo(dT) primer. The purified PCR product was cloned into the pGEM-T Easy vector, and the +1 site was identified from the DNA sequences.

OxyR purification and electrophoresis mobility shift assay. 6His-tagged OxyR from *P. aeruginosa* was purified using the pQE-30Xa expression system in a manner similar to that used for TtcA purification. The purity of OxyR was more than 90% as a 35 kDa major band observed in SDS-PAGE. EMSA was performed as previously described19 using a 32P-labeled probe containing the putative ttcA promoter. The probe was amplified with 32P-labeled BT4990 and BT4991 primers. Binding reactions consisting of labeled probe in 20μl of reaction buffer containing 20 mM Tris-HCl (pH 7.0), 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 μg ml−1 calf thymus DNA, 0.5 μg ml−1 poly[dI-dC], 50 μg ml−1 salmon sperm DNA, 100 mM DTT, and purified OxyR were incubated at 25°C for 30 min. Gel electrophoresis and visualization was done as previously described.

Statistics. Group data are presented as means ± standard deviation (SD). The Student’s t-test was used to determine differences between means using the function of Excel (Microsoft, Washington) and the SPSS (version 17.0; SPSS Inc.) statistical package. Unless otherwise stated, p values of <0.05 were considered significant.

Ethics statement. All *P. aeruginosa* and *D. melanogaster* were raised, maintained and all experiments were conducted following procedures, MUSC2016-002 and MUSC60-039-389, approved by the Committee of Biosafety, Faculty of Science, Mahidol University (MUSC) and the MUSC-Institutional Animal Care and Use Committee (IACUC), respectively.

References
1. Strzepe, A., Pritchard, K. A. & Dittel, B. N. Myeloperoxidase: A new player in autoimmunity. *Cell Immunol* 317, 1–8, https://doi.org/10.1016/j.cellimm.2017.05.002 (2017).
2. Dacovich, L. & Gorvel, J. P. Bacterial manipulation of innate immunity to promote infection. *Nat Rev Microbiol* 8, 117–128, https://doi.org/10.1038/nrmicro2295 (2010).
3. Li, Z. et al. Roles of reactive oxygen species in cell signaling pathways and immune responses to viral infections. *Arch Virology* 162, 603–610, https://doi.org/10.1007/s00705-016-1310-2 (2017).
4. Eiraty, B., Gennaris, A., Barras, F. & Collet, J. P. Oxidative stress, protein damage and repair in bacteria. *Nat Rev Microbiol* 15, 385–396, https://doi.org/10.1038/nrmicro.2017.26 (2017).
5. Staerck, C. et al. Microbial antioxidant defense enzymes. *Microb Pathog* 110, 56–65, https://doi.org/10.1016/j.micpath.2017.06.015 (2017).
6. Romsang, A. et al. The iron-sulphur cluster biosynthesis regulator IscR contributes to iron homeostasis and resistance to oxidants in *Pseudomonas aeruginosa*. *PLoS One* 9, e86763, https://doi.org/10.1371/journal.pone.0086763 (2014).
7. Romsang, A., Atchartpongkul, S., Trinacharinavit, W., Vattanavooboon, P. & Mongkol suk, S. Gene expression and physiological role of *Pseudomonas aeruginosa* methionine sulfoxide reductases during oxidative stress. *J Bacteriol* 195, 3299–3308, https://doi.org/10.1128/JB.00167-13 (2013).
8. Inlay, J. A. Transcription Factors That Defend Bacteria Against Reactive Oxygen Species. *Annu Rev Microbiol* 69, 93–108, https://doi.org/10.1146/annurev-micro-091014-104522 (2015).
9. Dubbs, J. M. & Mongkol suk, S. Peroxide-sensing transcriptional regulators in bacteria. *J Bacteriol* 194, 5495–5503, https://doi.org/10.1128/JB.00304-12 (2012).
10. Zheng, M. & Storz, G. Redox sensing by prokaryotic transcription factors. *Biochem Pharmacol* 59, 1–6 (2000).
11. Ochsner, U. A., Vasil, M. L., Alsabbagh, E., Parvatiyar, K. & Hassett, D. J. Role of the *Pseudomonas aeruginosa* oxyR-recG operon in oxidative stress defense and DNA repair: OxyR-dependent regulation of katB-ankB, ahpB, and ahpC-ahpF. *J Bacteriol* 182, 4533–4544 (2000).
12. Gu, M. & Inlay, J. A. The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide. *Mol Microbiol* 79, 1136–1150, https://doi.org/10.1111/j.1365-2958.2010.07520.x (2011).
13. Endres, L., Dedon, P. C. & Begley, T. J. Codon-biased translation can be regulated by wobble-base tRNA modification systems during cellular stress responses. *RNA Biol* 12, 603–614, https://doi.org/10.1080/15476286.2015.1031947 (2015).
14. Gu, C., Begley, T. J. & Dedon, P. C. tRNA modifications regulate translation during cellular stress. *FEBS Lett* 588, 4287–4296, https://doi.org/10.1016/j.febslet.2014.09.038 (2014).
15. Dedon, P. C. & Begley, T. J. A system of RNA modifications and biased codon use controls cellular stress response at the level of translation. *Chem Rev Toxicol* 27, 330–337, https://doi.org/10.1021/tx400438d (2014).
16. Shippy, D. C. & Fall, A. A. tRNA modification enzymes GidA and MnmE: potential role in virulence of bacterial pathogens. *Int J Mol Sci* 15, 18267–18280, https://doi.org/10.3390/ijms151018267 (2014).
17. Cho, K. H. & Caparon, M. G. tRNA modification by GidA/MnmE is necessary for Streptococcus pyogenes virulence: a new strategy to make live attenuated strains. *Infect Immun* 76, 3176–3186, https://doi.org/10.1128/IAI.01721-07 (2008).
18. Jaronensk, J. et al. Methylation at position 32 of tRNA catalyzed by TrmJ alters oxidative stress response in *Pseudomonas aeruginosa*. *Nucleic Acids Res* 44, 10854–10858, https://doi.org/10.1093/nar/gkw870 (2016).
19. Bouvier, D. et al. TtcA new tRNA-thioltransferase with an Fe-S cluster. *Nucleic Acids Res* 42, 7960–7970, https://doi.org/10.1093/nar/gku508 (2014).
20. Bork, P. & Koonin, E. V. A P-loop-like motif in a widespread ATP pyrophosphatase domain: implications for the evolution of sequence motifs and enzyme activity. *Proteins* 20, 347–355, https://doi.org/10.1002/pro.340200407 (1994).
Conceived and designed the experiments by A.R. Performed the experiments by A.R., J.D., K.K., L.W. and K.S. All authors reviewed the manuscript.

Acknowledgements

The authors gratefully acknowledge Wachareeporn Trinachartvanit, Soraya Pornsuwan and Siraphat Klowoothtipat for their excellent technical assistances. The authors thank James M. Dubbs for critical reading of the revised manuscript. This work was supported by grants from the Mahidol University, the Center for Emerging Bacterial Infections (EBI) and the Central Instrument Facility (CIF grant) of the Faculty of Science, Mahidol University, and Chulabhorn Research Institute. AR was supported by the grants from the Talent Management Program of Mahidol University (TM117/2557) and the joint funding of the Office of the Higher Education Commission and the Thailand Research Fund (MRG5980047), Thailand. LW were supported by grants from the Royal Golden Jubilee (RJ) Ph.D. Program (PHD/0132/2557). Parts of this work are from the thesis of KK submitted for the M.Sc. degree from Mahidol University.

Author Contributions

Conceived and designed the experiments by A.R. Performed the experiments by A.R., J.D., K.K., L.W. and K.S. Analyzed the data by A.R., M.F. and P.V. Contributed reagents/materials/analysis tools by A.R. and S.M. Wrote the paper by A.R. and SM. All authors reviewed the manuscript.
Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-30368-y.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018