Dual promoters of the major catalase (KatA) govern distinct survival strategies of Pseudomonas aeruginosa

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KatA is the major catalase required for hydrogen peroxide (H$_2$O$_2$) resistance and acute virulence in Pseudomonas aeruginosa PA14, whose transcription is driven from the promoter (katAp1) located at 155 nucleotide (nt) upstream of the start codon. Here, we identified another promoter (katAp2), the +1 of which was mapped at the 51 nt upstream of the start codon, which was responsible for the basal transcription during the planktonic culture and down-regulated upon H$_2$O$_2$ treatment under the control by the master regulator of anaerobiosis, Anr. To dissect the roles of the dual promoters in conditions involving KatA, we created the promoter mutants for each -10 box (p1m, p2m, and p1p2m) and found that katAp1 is required for the function of KatA in the logarithmic growth phase during the planktonic culture as well as in acute virulence, whereas katAp2 is required for the function of KatA in the stationary phase as well as in the prolonged biofilm culture. This dismantling of the dual promoters of katA sheds light on the roles of KatA in stress resistance in both proliferative and growth-restrictive conditions and thus provides an insight into the regulatory impacts of the major catalase on the survival strategies of P. aeruginosa.

Pseudomonas aeruginosa is a non-fermentative bacterial pathogen that respires on oxygen as well as nitrogen oxides, and its mode of respiration is likely associated with the infection strategies: P. aeruginosa is capable of rapid colonization primarily through aerobic respiration during acute infections, which involve various virulence factors; in contrast, P. aeruginosa survives persistently as a result of chronic infections promoted by biofilm mode of growth, which involve microaerobic or anaerobic respiration on nitrate (NO$_3^-$) under the hypoxic condition within the microbial population. Thus, the characteristics of both infection strategies that P. aeruginosa exploits are quite distinct and known to be associated with a subset of virulence or persistence factors that should be orchestrated to support the survival of P. aeruginosa during each mode of interaction with the host environments. For example, invasive functions such as secreted toxins and adhesion factors are required for acute virulence, but detrimental for chronic infection or persistence, depending on their cost-benefits. This is demonstrated by accumulation of the mutations in the invasive functions over time, once infection is established.

We previously reported the role of the major catalase, KatA in peroxide resistance, osmoprotection, and acute virulence of a P. aeruginosa strain. Unlike other major bacterial catalases, KatA is highly stable and found in the extracellular milieu, which ensures the survival of P. aeruginosa cells in their biofilm. The basal expression of KatA is relatively high, but its expression is further increased upon stationary growth phase and in response to H$_2$O$_2$ under the control of OxyR, the master regulator in response to H$_2$O$_2$. OxyR normally binds to the upstream region of the katA promoter as a functional repressor and switches to the activator upon H$_2$O$_2$ challenge, where C199 has a critical role in both functions and in acute virulence. Recently, KatA was shown to play a critical role in nitric oxide buffering under anaerobic respiration conditions, upon which KatA expression was increased by Anr, the master regulator of anaerobiosis required for dissimilatory NO$_3^-$ reduction. It has been suggested by Trunk et al. that KatA may belong to the Anr regulon, with a presumable Anr box located upstream of the KatA initiation codon. Although much has been being unveiled about the physiological roles of KatA and the regulatory mechanisms that involve OxyR and its cis-element of the katA promoter, more needs to be clarified to...
account for the involvement of Anr and the elevated expression of KatA upon anaerobic respiration for its roles to ensure the distinct infection strategies of *P. aeruginosa*.

In the present study, we have explored the regulatory module underlying the anaerobic regulation of KatA, by newly identifying a second promoter (*katAp2*) of the *katA* gene, which is responsible for the basal expression of KatA during the planktonic growth of *P. aeruginosa* strain PA14. This second promoter is regulated primarily by Anr. More importantly, the mutation analyses of each promoter revealed the separate roles of the dual promoters to cope with reactive oxygen and/or nitrogen species (ROS and RNS), which is dependent on the growth states.

### Results

**Identification of a second promoter (*katAp2*) of the *katA* gene.** A transcription start site of the *katA* gene was identified at the 155 nucleotide (nt) upstream (T-155) of the *katA* initiation codon, whose transcription is induced upon H$_2$O$_2$ treatment. Despite over 5-fold induction of *katAp1* transcription upon H$_2$O$_2$ exposure, the *katA-lacZ* fusion displayed a minor (~2-fold) induction, which may be attributed to another level of regulation. Based on this speculation, we have identified another promoter of the *katA* gene, *katAp2*: from the high-resolution S1 nuclease mapping data, a second protective band was observed at the 51 nt upstream (T-51) of the translation initiation codon (Fig. 1A). The upstream of the position of T-51 could be a potential promoter by its context. It is of marked interest that the putative Anr box consensus (TTGac-N4-gtCAA) was identified, which overlaps with the potential −35 box of *katAp2* (Fig. 1B), since KatA expression is deemed reduced in the *anr* mutant as previously assessed by the transcriptome and proteome analyses.

Identification of the *katAp2* promoter and the potential Anr box suggests that the *anr*-dependent regulation of KatA might be most likely attributed to this second promoter. Moreover, the *katAp2* transcription was slightly reduced upon H$_2$O$_2$ treatment (Fig. 1A; see below), which is in a good agreement with the fact that the Fe-S center of Anr is vulnerable to ROS and RNS with its transactivation activity compromised in the presence of O$_2^-$, H$_2$O$_2$, and NO$^\cdot$.

**Regulation of *katAp2* by Anr during aerobic NO$_3^-$ respiration.** To verify the involvement of Anr in the regulation of *katAp2*, we examined the *katA* transcription in the *anr* null mutant, by using low-resolution S1 nuclease protection assay (Fig. 2A). The *katAp1* transcription was highly elevated, whereas the *katAp2* transcription was slightly reduced upon H$_2$O$_2$ treatment in the wild type bacteria. In contrast, however, the *katAp2* transcription in the *anr* mutant was significantly reduced even in the absence of H$_2$O$_2$ treatment and the *katAp1*
transcription was not affected in the anr mutant. Furthermore, the H2O2-mediated decrease in katA-driven transcription is not associated with OxyR, in that the katA transcription pattern in the oxyR null mutant did not significantly differ from that in the wild type bacteria. This result suggests that the dual promoters of katA were regulated separately by two global regulators, OxyR and Anr.

Since the katA promoter is abolished in the anr mutant, accounting for the basal expression of KatA during the planktonic growth, we have tested if the Anr-dependent NO3− respiration during the planktonic growth may be associated with the katA transcription as well. We have exploited the mutants for the genes encoding another downstream regulator, Dnr and the four nitrogen oxide reductases (Nar, Nir, Nor, and Nos) involved in dissimilatory denitrification16: NO3− is reduced to nitrite (NO2−) by Nar; NO2− is reduced to NO by Nir; NO is reduced to N2O by Nor; N2O is reduced to N2 by Nos. Denitrification is vital for growth and survival under microaerobic and anaerobic conditions as found in biofilms and microcolonies.

We found that only the nirS mutant displayed reduced katA transcription (Figs 2B and S1). This is in good agreement with the previous observation by Su et al.11 that NO and Anr might be required for the increased KatA activity under anaerobic conditions.

Creation of the katA promoter mutants. To explore the roles of the dual promoters in the expression of KatA that is required for several phenotypes such as stress resistance and virulence in P. aeruginosaa, we created the promoter mutants for each presumable promoter 10 box, as described in Fig. 3A. The substitution mutations were introduced into the chromosome by pEX18T-based allelic exchange, which was confirmed by PCR followed by restriction enzyme digestions. The functional verification of the created promoter mutants (p1m, p2m and p1p2m) was performed based on the expression patterns and catalase activity profiles in the mutants (Fig. 3B,C). The katAp1 and katAp2 transcripts from the promoter mutants were analyzed by S1 nuclease protection assay, which verified that the introduced substitutions at the proposed −10 box resulted in the specific loss of the corresponding transcripts. A slower migrating band that was newly observed by the p2m mutation did not affect the catalase profiles in the mutants (Fig. 3C). As a result, the basal expression of KatA during the planktonic growth was abolished by the p2m mutation throughout the entire growth phases (Fig. 3D), whereas the p1m mutation contributed only to the induced expression upon H2O2 treatment, which was more evident in the p2m mutant (Figs. 3C and S2). These results indicate that the katAp2 promoter is involved in the basal as well as increased expression during the planktonic growth, whereas katAp1 is responsible for the H2O2-inducible expression of KatA. Because the katAp2 requires the transactivator, Anr for its transcription, we have postulated that Anr is somehow active even in the aerobic planktonic culture in complex media such as PIA that contains ~63 μM NO3− used for NO3− respiration17 (Fig. 2). However, as shown in Fig. S3, the stationary phase-induced transcription of katAp2 was not completely abolished in the double mutant for anr and rpoS, indicating that it involves more complicated regulatory networks such as quorum sensing and so on19.

katAp1 is critical in the well-growing state, whereas katAp2 is critical in the growth-restricting state. We first investigated the several stress-related phenotypes of the promoter mutants in comparison with the katA null mutant, which is hypersusceptible to H2O2 or acidified nitrite (aNO2)20,21,22. As shown in Fig. 4A, when the cells were tested during the logarithmic growth phase, the p1m mutant and the p1p2m mutant were hypersusceptible to H2O2 or aNO2 treatment, whereas the p2m mutant was no more susceptible than the wild type. In contrast, however, when cells were taken during the stationary growth phase or from the biofilm culture, the outcome did clearly differ: the p2m mutant as well as the p1p2m mutant was hypersusceptible to H2O2 or aNO2 treatment, whereas the p1m mutant was no more susceptible than the wild type (Fig. 4B,C). These results may be associated with the inducible expression of KatA by katAp1 when the basal expression of KatA by katAp2 might be...
amended with 15 mM KNO₃. Culture aliquots were taken at every 2 h from 2 to 10 h post-inoculation and then subjected to low-resolution S1 nuclease assay. The two transcriptional start sites of the katA gene are indicated by open (katA1) and closed (katA2) arrows. (C) Catalase activity staining in cell extracts of the wild type (WT) and the katA null and promoter mutant (katAΔ, p1m, p2m, and p1p2m) cells with (+) or without (−) 1 mM H₂O₂ treatment for 10 min at OD₆₀₀ of 0.5 in LB were subjected to low-resolution S1 nuclease assay. The two transcriptional start sites of the katA gene are indicated by open (katA1) and closed (katA2) arrows. (D) Promoter activity during aerobic planktonic growth. The wild type cells harboring one of the promoter transcription fusions (●, WT; ◇, p1m; □, p2m; △, p1p2m) were grown in LB amended with 15 mM KNO₃. Culture aliquots were taken at every 2 h from 2 to 10 h post-inoculation and then subjected to β-galactosidase (LacZ) assay. The data represent the average of the means of three independent experiments (two cultures per experiment), with the error bars representing the standard deviations.

**Figure 3.** Creation of the katA promoter mutants. (A) Schematic representation. The katA gene and its potential promoter elements (−35 and −10 boxes) are designated. The sequences of each −10 box are indicated above the boxes. The katA promoter mutations were constructed by substituting the −10 box with the KpnI site (GGTACC) for the katA1 mutant (p1m) or the BamHI site (GGATCC) for the katA2 mutant (p2m) as indicated with the mutated nucleotides underlined. A double mutant for both promoters (p1p2m) was generated as well. (B) Transcription upon H₂O₂ induction. Total RNA (50 µg) isolated from the wild type (WT) and the katA promoter mutant (p1m, p2m, and p1p2m) cells with (+) or without (−) 1 mM H₂O₂ treatment for 10 min at OD₆₀₀ of 0.5 in LB were subjected to low-resolution S1 nuclease assay. The two transcriptional start sites of the katA gene are indicated by open (katA1) and closed (katA2) arrows. (C) Catalase activity staining in cell extracts of the wild type (WT) and the katA null and promoter mutant (katAΔ, p1m, p2m, and p1p2m) cells with (+) or without (−) 1 mM H₂O₂ treatment for 10 min as in (B) were visualized using 50 µg of proteins in each cell extract. The two catalase bands are indicated by open (KatB) and closed (KatA) arrows. (D) Promoter activity during aerobic planktonic growth. The wild type cells harboring one of the promoter transcription fusions (●, WT; ◇, p1m; □, p2m; △, p1p2m) were grown in LB amended with 15 mM KNO₃. Culture aliquots were taken at every 2 h from 2 to 10 h post-inoculation and then subjected to β-galactosidase (LacZ) assay. The data represent the average of the means of three independent experiments (two cultures per experiment), with the error bars representing the standard deviations.

**katA1, but not katA2, contributes to acute virulence.** We next measured the virulence potential of the promoter mutants using Drosophila and murine acute infection models, since the katA null mutant of PA14 strain was virulence-attenuated in the same infection models⁶. It is of marked interest that p1m but not p2m displayed virulence attenuation in both infection models (Fig. 5), suggesting that the inducible KatA expression driven by katA1 is critical in the host environments. Although we do not have any direct evidence on whether the katA2 transcription would occur during the infection conditions, it is clear that the katA2-mediated KatA expression is likely dispensable in the virulence pathways at least under our experimental conditions.

**katA2 contributes to lowering endogenously generated RNS.** We hypothesized that KatA might have some surveillance function for homeostatic maintenance of cellular states regarding ROS and RNS by lowering the levels of endogenously generated ROS and RNS. We speculated that ROS and/or RNS could be generated as well. (B) Transcription upon H₂O₂ induction. Total RNA (50 µg) isolated from the wild type (WT) and the katA promoter mutant (p1m, p2m, and p1p2m) cells with (+) or without (−) 1 mM H₂O₂ treatment for 10 min as in (B) were visualized using 50 µg of proteins in each cell extract. The two catalase bands are indicated by open (KatB) and closed (KatA) arrows. (D) Promoter activity during aerobic planktonic growth. The wild type cells harboring one of the promoter transcription fusions (●, WT; ◇, p1m; □, p2m; △, p1p2m) were grown in LB amended with 15 mM KNO₃. Culture aliquots were taken at every 2 h from 2 to 10 h post-inoculation and then subjected to β-galactosidase (LacZ) assay. The data represent the average of the means of three independent experiments (two cultures per experiment), with the error bars representing the standard deviations.

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generation of RNS, under assumption that the NO₃⁻ respiration activity of the katA mutant does not significantly differ from that of the wild type bacteria, which requires more extensive investigation.

Discussion
KatA is the major catalase of P. aeruginosa, whose “adaptive” function is to dismutate H₂O₂, an ROS either sporadically generated during the normal respiration on O₂ or externally provided by redox-cycling agents or host factors. However, KatA exhibited higher activity under anaerobic conditions than aerobic conditions[23], where no ROS could be potentially generated. This “enigmatic” expression profile of KatA under anaerobic conditions has been granted with the evidence that the KatA expression is lower in the anr mutant than in the wild type, with the potential Anr box located upstream of the katA coding region[12]. However, it had not been fully substantiated until the study about KatA function in anaerobiosis was reported[11]. They found that KatA contributes to the resistance to aNO₂ that generates RNS like NO. They also proposed that KatA binds to NO to sequester and prevent it from its harmful effect, a new role of KatA in buffering free NO to ensure P. aeruginosa anaerobiosis, based on the investigation of direct NO-KatA interaction under anaerobic condition, as first demonstrated in bovine liver catalase able to complex with NO at the heme[24]. Although the anaerobic KatA expression could be justified by its proposed function, the exact regulatory mechanisms have remained elusive. In this study, we first connect the dots between the previous results regarding the expression and the function of KatA under anaerobic conditions, by identifying the second promoter, katAp2. This promoter is positively regulated by Anr and required to lower the endogenous generation of RNS during planktonic culture, which we now call the “surveillance” function of KatA.

Figure 4. Stress resistance of the katA promoter mutants. (A,B) Stress resistance in planktonic culture. Susceptibility to H₂O₂ and acidified NaNO₂ (aNO₂) were assessed for the wild type (WT) and the katA null and promoter mutant (katA, p1m, p2m, and p1p2m) cells that had been cultured for 3 h or for 6 h in LB broth (pH 6.5), and treated for 24 h with either 15 mM H₂O₂ and 200μM NaNO₂ or in LB broth (pH 6.5) (−). Ten-fold serial cell dilutions from the 3-h (A; logarithmic) and 6-h (B; stationary) cultures were spotted on LB agar plate. The numbers indicate the log (cfu) of the applied bacterial spots. (C) Stress resistance in static culture. The wild type (WT) and the katA null and promoter mutant (katA, p1m, p2m, and p1p2m) cells were statically and anaerobically cultured for 7 days in LB broth (pH 6.5) treated for 1 h with either 100 mM H₂O₂ and 1.2 M NaNO₂, or in LB broth (pH 6.5) (−). Ten-fold serial cell dilutions from the cultures were spotted on LB agar plate. The numbers indicate the log(cfu) of the applied bacterial spots.

In our previous study, P. aeruginosa KatA exhibits unusual properties ascribed to its metastability, high specific activity, and/or extracellular presence, unlike the other clade 3 monofunctional bacterial catalases such as Streptomyces coelicolor CatA and Bacillus subtilis KatA (BsKatA)[7]. At least one of those properties has been proposed to be implicated in biofilm life style of P. aeruginosa. P. aeruginosa respires on NO₂⁻ and/or NO₃⁻ under anaerobic conditions, which is in marked contrast to the two bacterial species: S. coelicolor is unable to grow under anaerobic conditions; B. subtilis is capable of fermentation under anaerobic conditions. Interestingly, BsKatA is subjected to activity modulation called “instant adaptation” by NO in response to ROS[11,25]. Although the careful comparison between KatA and the related catalases needs to be furthered in regards to the physiological traits of the cognate bacteria, the functions of KatA in the microaerobic and anaerobic life style of P. aeruginosa may
be attributed to the unusual biochemical properties of KatA, most likely the extracellular presence, as previously suggested. This aspect will open a new venue for an insight into molecular evolution of a clade 3 bacterial catalase to fulfill the new needs that the bacterium is faced with, by delving into the roles of the particular amino acid residues of KatA associated with the extracellular and/or periplasmic presence and the protective function to buffer the NO, because NO is normally generated in the periplasm by Nir during microaerobic and/or biofilm growth of \( P. \) aeruginosa.

Another important contribution of this work is to provide a new picture to associate the protective functions of KatA with its regulation governed by OxyR and Anr. It should be noted that OxyR and Anr work apparently under the opposite conditions: for example, OxyR is activated by H\(_2\)O\(_2\) or NO\(_2\), while Anr is inactivated by H\(_2\)O\(_2\) or NO, because its Fe-S cluster is vulnerable to H\(_2\)O\(_2\) and NO. Nevertheless, the dual promoters ensure the \( katA \) transcription in both balanced (i.e. surveillance function-requiring) and perturbed (i.e. adaptive function-requiring) conditions of ROS and/or RNS homeostasis. It is also likely that OxyR and Anr may cooperate for proper KatA expression during infection, due to the complicated respiration mode for \( P. \) aeruginosa growth that would be microaerophilic along the continuum between aerobic and anaerobic conditions in the human airways.

Finally, the present study clearly demonstrates the discrete roles of the dual promoters in the KatA-associated phenotypes. The creation of the chromosomal mutations for the presumable promoters worked well to disrupt each promoter activity and the subsequent KatA expression specifically from the corresponding promoters. Use of these promoter mutants helped clarify the roles of each promoter in the functions of KatA in \( P. \) aeruginosa physiology. Based on these properties regarding regulation and function, the pivotal roles of KatA during the growth and survival of \( P. \) aeruginosa warrant further verification. This can be done by using the promoter mutants created in the present study. Moreover, the complex involvement of multiple regulatory systems will be comprehensively

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**Figure 5. Acute virulence of the \( katA \) promoter mutants.** Virulence in \( Drosophila \) (A) and mouse peritoneal (B) infection models. The wild type (WT) and the mutant (\( katA \), \( p1m \), \( p2m \), and \( p1p2m \)) cells were prepared and the survival of infected animals was determined as described in Methods. The values are the averages from five replicate experiments for \( Drosophila \) (A) and three for mouse (B) infections. Statistical significance based on the log-rank test is indicated (**\( p < 0.005 \)).

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**Figure 6. Accumulation of NO\(_2^-\) in the \( katA \) promoter mutants.** Steady-state level of extracellular NO\(_2^-\) was measured in the wild type (WT) and the \( katA \) null and promoter mutant (\( katA \), \( p1m \), \( p2m \), and \( p1p2m \)) cells as well as \( anr \) mutant cells that had been grown to OD\(_{600}\) of 0.7. The amount (\( \mu M \)) of NO\(_2^-\) is calculated using the standard curve (r\(^2\) = 0.999) and the average values measured from the three independent experiments, with the error bars representing the one positive value of the standard deviations. Statistical significance based on the Student's t-test is indicated (**\( p < 0.01 \); ***\( p < 0.005 \)).
explored at various regulatory levels, especially in response to stationary phase, quorum-sensing, and/or iron availability etc. All these aspects that will be more directly elucidated may provide a new insight into the therapeutic targets of this and the related bacteria associated with the stress responses toward ROS and RNS.

Methods

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* strains were grown at 37 °C using Luria-Bertani (LB) broth or on 1.5% Bacto-agar solidified LB plates. For anaerobic growth, bacteria were grown in LB medium supplemented with 15 mM KNO₃ in an anaerobic jar with AnaeroPack (MGC). Overnight-grown cultures were used as inoculum (1.6 × 10⁷ cfu/ml) into fresh LB broth and grown at 37°C to the OD₆₀₀ as indicated and then used for the experiments described herein.

DNA oligonucleotide primers. The information on DNA oligonucleotide primers used for gene deletion, expression, and detection in this study are listed in Table S1.

RNA isolation and S1 nuclease protection analysis. *P. aeruginosa* strains were grown in LB or LB containing 15 mM KNO₃ media and then the half of the culture was treated with 1 mM H₂O₂ for 10 min, with the remaining half used as the untreated control. Total RNA was isolated from 10⁶ cells by using RNeasy kit (Qiagen) and 50 μg of RNA samples were used for S1 nuclease protection experiment as described elsewhere²⁶. Briefly, the PCR-generated probe using the oligonucleotide primer pairs (katA-N10: 5’ end at −133 and katA-S1C1: 5’ end at +264) was labeled with [γ-³²P] ATP by T4 polynucleotide kinase. For hybridization, the mixtures of RNA samples and labeled probe were incubated at 90 °C for 10 min for denaturation, and then slowly cooled down to 55 °C for hybridization. After overnight hybridization, S1 nuclease digestion was performed at 30 °C for 30 min by adding 8 units of S1 nuclease for each sample. The reaction was stopped and precipitated with 100% ethanol. The samples were dissolved and denatured at 90 °C for 5 min in formamide-dye solution, and then analyzed by 6% PAGE containing 7 M urea. For high resolution S1 mapping, the unlabelled katA-S1C1 was used to generate the nucleotide sequence ladder using Sequenase Version 2.0 DNA Sequencing Kit (USB) with [³²P] dATP and pUCP-katA as the template²⁷.

Creation of the katA promoter mutants. The katA promoter mutant allele for the katAp1 (−10 box; CATCCCT to GGTACC, the KpnI site) or for the katAp2 (−10 box; CACGCT to GGATCC, the BamHI site) were generated by SOEing (splicing by overlap extension) PCR using 4 oligonucleotide primers. The resulting PCR products were cloned into pEX18T. These katA promoter mutant alleles were introduced into PA14 chromosome as described elsewhere²⁸.

Creation of the lacZ fusions and β-galactosidase assay. All the lacZ fusions of the katA promoter regions were created by SOEing PCR using 4 oligonucleotide primers (Table S1). The primers were designed to generate transcriptional fusions of the katA promoter regions without its own ribosome-binding site (RBS) at the upstream of the lacZ RBS. Briefly, the katA promoter regions were prepared by using the primers pairs (katA-N3 and katA-lacZ-UC) and each of the chromosomes from the WT and the promoter mutant (p1m, p2m and p1p2m) bacteria as the templates. The lacZ coding region was generated by using the primer pairs (katA-lacZ-DN and pQF50-lacZ-C1) and the pQF50 plasmid as the template. The PCR products were fused by SOEing PCR using katA-N3 and pQF50-lacZ-C1 primers and the amplified fragments were cloned into pQF50. These promoter fusion constructs were introduced by electroporation and LacZ (β-galactosidase) activity was determined using the bacterial culture aliquots taken at the indicated time points as previously described³⁰.

Catalase activity staining. Catalase activity staining was performed as described previously²⁷. Briefly, cell extracts (40 μg) were applied to a 7% native polyacrylamide gel and electrophoresed. The gel was washed in distilled water and then treated with 1 mM H₂O₂ for 10 min. After treatment, the gel was rinsed and transferred to 1% (w/v) ferric chloride and 1% (w/v) potassium ferricyanide solution. The reaction was stopped by washing in distilled water.

Stress susceptibility measurement. Stress susceptibility was measured based on the survival of the *P. aeruginosa* cells upon 24 h treatment to 15 mM H₂O₂ or 200 μM NaNO₂ for prolonged exposure. For pulse-treatment, cells were exposed to 100 mM H₂O₂ or 1.2 mM NaNO₂ in acidified LB (pH 6.5). After exposure, 10-fold serial dilutions (3 μl) of the cells were spotted onto an LB agar plate to enumerate the survivor bacteria.

Virulence measurement. Determination of mortality from *Drosophila* systemic infections and mouse peritonitis-sepsis caused by *P. aeruginosa* cells that had been grown to the OD₆₀₀ of 3.0 was performed as previously described³⁰. For *Drosophila* systemic infections, 3- to 6-day-old adult female flies (Oregon R) were infected by pricking at the dorsal thorax with a 10 mm needle (Ernest F. Fullam, Inc.). The needle dipped into bacterial suspensions diluted in 10 mM MgSO₄ containing 10⁷ cfu. Fly mortality was monitored for 54 h postinfection. For murine peritonitis-sepsis, bacterial cells were harvested, washed twice with phosphate buffered saline (PBS) (2.7 mM KCl, 137 mM NaCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄ pH 7.0) and diluted to 2 × 10⁶ cfu in 100 ml of PBS containing 1% mucin as an adjuvant. Anesthetized 4-week-old mice (ICR) were intraperitoneally infected according to the institutional protocol approved by the Institutional Animal Care and Use Committee at CHA University. Kaplan-Meier analysis and log-rank tests were used to compare the virulence difference between the groups³⁰,³¹. A p value of less than 0.01 was considered to be significant.
Table 1. Bacterial strains and plasmids used in this study. *Rif<sup>+</sup>, rifampicin-resistant; Gm<sup>+</sup>, gentamicin-resistant; Ch<sup>+</sup>, carbenicillin- and ampicillin-resistant; Tp<sup>+</sup>, trimethoprim-resistant; Sm<sup>+</sup>, streptomycin-resistant. †underlines; mutated nucleotides at the presumably 10 boxes.

| Strain or plasmid | Relevant characteristics or purpose<sup>a</sup> | Reference or source |
|-------------------|---------------------------------------------|---------------------|
| Strains          |                                             |                     |
| PA14             | wild type laboratory strain; Rif<sup>+</sup> | Lab collection      |
| katA             | PA14 with in-frame deletion of katA; Rif<sup>+</sup> | Lee et al.†        |
| oxyR             | PA14 with in-frame deletion of oxyR; Rif<sup>+</sup> | Choi et al.†       |
| anr              | PA14 with in-frame deletion of anr; Rif<sup>+</sup> | This study          |
| rpoS             | PA14 with in-frame deletion of rpoS; Rif<sup>+</sup> | Park et al.†      |
| anrrpS           | anr with in-frame deletion of rpoS; Rif<sup>+</sup> | This study          |
| natG             | PA14 with MAR2 × 77 insertion at natG; Rif<sup>+</sup>, Gm<sup>+</sup> | Liberati et al.‡  |
| natS             | PA14 with MAR2 × 77 insertion at natS; Rif<sup>+</sup>, Gm<sup>+</sup> | Liberati et al.‡  |
| norB             | PA14 with MAR2 × 77 insertion at norB; Rif<sup>+</sup>, Gm<sup>+</sup> | Liberati et al.‡  |
| nosZ             | PA14 with MAR2 × 77 insertion at nosZ; Rif<sup>+</sup>, Gm<sup>+</sup> | Liberati et al.‡  |
| dnr              | PA14 with MAR2 × 77 insertion at dnr; Rif<sup>+</sup>, Gm<sup>+</sup> | Liberati et al.‡  |
| p1m              | PA14 with the chromosomal mutation (CACGCT to GGATCC)<sup>b</sup> at the −10 box of katAp<sub>2</sub>; Rif<sup>+</sup> | This study          |
| p2m              | PA14 with the chromosomal mutation (CACGCT to GGATCC)<sup>b</sup> at the −10 box of katAp<sub>2</sub>; Rif<sup>+</sup> | This study          |
| p1p2m            | p1m with the chromosomal mutation (CACGCT to GGATCC)<sup>b</sup> at the −10 box of katAp<sub>2</sub>; Rif<sup>+</sup> | This study          |
| E. coli          |                                            |                     |
| DH5<sup>Δ</sup>          | multi-purpose cloning                      | Lab collection      |
| S17-1            | conjugal transfer of mobilizable plasmid; Tp<sup>+</sup>, Sm<sup>+</sup> | Lab collection      |
| Plasmids         |                                            |                     |
| pEX18T           | Positive selection suicide vector for allelic exchange; Ch<sup>+</sup> | Lab collection      |
| pEX18T-Δanr      | pEX18T with the in-frame deletion in the anr gene; Ch<sup>+</sup> | This study          |
| pEX18T-katAp1m   | pUCP18 with the −10 box mutation (CATCCCT to GGATCC)<sup>c</sup> of katAp<sub>1</sub>; Ch<sup>+</sup> | This study          |
| pEX18T-katAp2m   | pUCP18 with the −10 box mutation (CATCCCT to GGATCC)<sup>c</sup> of katAp<sub>2</sub>; Ch<sup>+</sup> | This study          |
| pQF50-katAp      | lacZ transcriptional fusion; Ch<sup>+</sup> | Farinha and Kropinski<sup>†</sup> |
| pQF50-katAp<sup>d</sup> | pQF50 with the katA promoter; Ch<sup>+</sup> | This study          |
| pQF50-katAp1m    | pQF50 with the −10 box mutation (CATCCCT to GGATCC)<sup>c</sup> of katAp<sub>1</sub>; Ch<sup>+</sup> | This study          |
| pQF50-katAp2m    | pQF50 with the −10 box mutation (CATCCCT to GGATCC)<sup>c</sup> of katAp<sub>2</sub>; Ch<sup>+</sup> | This study          |
| pQF50-katAp1p2m  | pQF50 with the −10 box mutation (CATCCCT to GGATCC)<sup>c</sup> of katAp<sub>1</sub> and (CACGCT to GGATCC)<sup>d</sup> of katAp<sub>2</sub>; Ch<sup>+</sup> | This study          |

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**NO<sub>2</sub>⁻ measurement.** The steady-state level of NO<sub>2</sub>⁻ that had been endogenously generated and transported during the normal growth in LB amended with 15 mM KNO<sub>3</sub> was measured based on Griess reaction. Culture aliquots (300 μl) of the cells that had been grown to OD<sub>600</sub> of 0.7 were mixed with the equal volume of Griess reagent (Sigma, USA) and incubated at 37 °C for 10 min. The NO<sub>2</sub>⁻ level in the supernatant was measured by the absorbance at 550 nm. Statistical significance between the groups is indicated, based on a p value of less than 0.01 by the Student's t test.

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