Li et al. Journal of Biomedical Science (2020) 27:37
https://doi.org/10.1186/s12929-020-00631-4

Journal of Biomedical Science

MOST 科技部 Ministry of Science and Technology
The cost of publication in Journal of Biomedical Science is borne by the Ministry of Science and Technology, Taiwan.

RESEARCH Open Access

Protection from hydrogen peroxide stress relies mainly on AhpCF and KatA2 in *Stenotrophomonas maltophilia*

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Abstract

Background: Aerobically-grown bacteria can be challenged by hydrogen peroxide stress from endogenous aerobic metabolism and exogenously generated reactive oxygen species. Catalase (Kat), alkyl hydroperoxidase (Ahp), and glutathione peroxidase (Gpx) systems are major adaptive responses to H_2O_2 stress in bacteria. *Stenotrophomonas maltophilia* is a ubiquitous Gram-negative bacterium equipped with four Kats (KatA1, KatA2, KatMn, and KatE), one Ahp (AhpCF), and three Gpxs (Gpx1, Gpx2, and Gpx3). Here, we systematically investigated how the eight H_2O_2 scavenging genes differentially contribute to the low-micromolar levels of H_2O_2 generated from aerobic metabolism and high-millimolar levels of H_2O_2 from exogenous sources.

Methods: Gene expression was assessed and quantified by reverse transcription-PCR (RT-PCR) and real time quantitative PCR (qRT-PCR), respectively. The contribution of these enzymes to H_2O_2 stress was assessed using mutant construction and functional investigation.

Results: Of the eight genes, *katA2*, *ahpCF*, and *gpx3* were intrinsically expressed in response to low-micromolar levels of H_2O_2 from aerobic metabolism, and the expression of *katA2* and *ahpCF* was regulated by OxyR. AhpCF and KatA2 were responsible for alleviating aerobic growth-mediated low concentration H_2O_2 stress and AhpCF played a critical role for stationary-phase cells. *KatA2* was upregulated to compensate for AhpCF in the case of *ahpCF* inactivation. After exposure to millimolar levels of H_2O_2, *katA2* and *ahpCF* were upregulated in an OxyR-dependent manner. KatA2 was the critical enzyme for dealing with high concentration H_2O_2. Loss-of-function of KatA2 increased bacterial susceptibility to high concentration H_2O_2.

Conclusions: AhpCF and KatA2 are key enzymes protecting *S. maltophilia* from hydrogen peroxide stress.

Keywords: *Stenotrophomonas maltophilia*, Catalase, Alkyl hydroperoxidase, Glutathione peroxidase, Hydrogen peroxide stress, OxyR regulator

Background

In aerobic bacteria, hydrogen peroxide (H_2O_2) stress is endogenously generated by aerobic metabolism. Exogenous H_2O_2 stress can be generated by chemical processes, competing organisms, and host cells in the environment. Superoxide, H_2O_2, and hydroxyl radicals are three main reactive oxygen species (ROS) in aerobic bacteria. Unlike superoxide and hydroxyl radicals, H_2O_2 is not a free radical and is less toxic to bacteria. However, distinct from superoxide and hydroxyl radicals, H_2O_2 can easily diffuse across cell membranes. Furthermore, hydroxyl radical is the most reactive ROS species and it can be readily generated from H_2O_2 in the presence of Fe^{2+} via the Fenton reaction, causing irreversible damage to bacteria [1]. Therefore, effective removal of H_2O_2 is critical for bacterial survival.

To prevent H_2O_2-mediated damage, aerobic bacterial pathogens must quickly convert H_2O_2 into other, less dangerous substances. The most common and efficient

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systems for bacteria to alleviate H_2O_2 stresses are an array of scavenging enzymes [2], including catalase (Kat), glutathione peroxidase (Gpx), and alkyl hydroperoxidase/alkyl hydroperoxide reductase (Ahp) [3]. Catalase directly catalyzes the decomposition of hydrogen peroxide without oxidizing the enzyme itself. Peroxidases detoxify H_2O_2 by oxidizing itself and rely on cellular reductants to revive them from the oxidized state. A bacterium can harbor an array of H_2O_2 scavenging enzymes, like KatG, KatE, AhpCF, and BtuE in *E. coli* [4], and KatA, KatB, KatC, AhpA, AhpB, AhpCF, and BtuE in *P. aeruginosa* [5]. The H_2O_2 scavenging enzymes may differentially function in response to different oxidative stress sources.

OxyR, a LysR family transcription factor, is a well-characterized regulator of the H_2O_2 response in Gram-negative bacteria [6]. OxyR contains a regulatory domain and a DNA binding domain. After sensing a H_2O_2 threat, OxyR undergoes secondary structure rearrangement by forming a disulfide bond between the two conserved cysteine residues in the regulatory domain, resulting in oxidized OxyR. The oxidized OxyR binds to the promoter region of the target gene via the DNA binding domain, modulating target gene expression as a transcriptional activator or repressor.

Stenotrophomonas maltophilia is an aerobic, Gram-negative, γ-proteobacterium that is widely distributed in the soil, water, plant rhizosphere, and hospital equipment [7]. It is also a pathogen that infects cystic fibrosis and immunocompromised patients [8]. Because of its diverse habitats, *S. maltophilia* is expected to be equipped with more effective H_2O_2 alleviation systems to adapt to different environmental niches. Analysis of the *S. maltophilia* genome sequence indicates the presence of many H_2O_2 scavenging enzymes, including four distinct Kats, three Gpxs, and one alkyl hydroperoxidase/alkyl hydroperoxide reductase system (AhpCF) [9]. Given that three systems contribute to neutralize H_2O_2 stresses, a defect in a single system can be compensated by the others. Therefore, a global investigation of the three systems, instead of focusing on one system, is likely to contribute more to our understanding of H_2O_2 detoxification in bacteria. To our knowledge, no previous studies have comprehensively investigated the function and interplay among the three antioxidant systems in *S. maltophilia*. This study aimed to provide this information and elucidate the role of these antioxidant enzymes in protecting bacteria against H_2O_2 stress from aerobic metabolism or exogenous sources.

Methods

Bacterial strains, plasmid, and growth condition

Table S1 lists the bacterial strains, plasmids, and PCR primers used in this study. All primers used in this study were designed based on the genome of *S. maltophilia* K279a.

Construction of in-frame deletion mutants

The strategy of two-step double cross-over homologous recombination was used for the construction of mutants used in this study. Two PCR amplicons, corresponding to upstream and downstream of the gene intended to delete, were amplified using the paired primer sets and subsequently cloned into pEX18Tc to yield the recombinant plasmids for mutants construction. The primer sets used are KatA1N-F/KatA1N-R and KatA1C-F/KatA1C-R for plasmid pΔKatA1, KatA2N-F/KatA2N-R and KatA2C-F/KatA2C-R for plasmid pΔKatA2, KatMnN-F/KatMnN-R and KatMnC-F/KatMnC-R for plasmid pΔKatMn, KatEN-F/KatEN-R and KatEC-F/KatEC-R for plasmid pΔKatE, AhpCN-F/AhpCN-R and AhpFC-F/AhpFC-R for plasmid pΔAhpCF, Gpx1N-F/Gpx1N-R and Gpx1C-F/Gpx1C-R for plasmid pΔGpx1, Gpx2N-F/Gpx2N-R and Gpx2C-F/Gpx2C-R for plasmid pΔGpx2, and Gpx3N-F/Gpx3N-R and Gpx3C-F/Gpx3C-R for plasmid pΔGpx3 (Table S1). These pEX18Tc-derived plasmids were mobilized into KJ cells by conjugation and the transconjugants selection were performed as descried previously [10]. PCR and DNA sequencing were performed to confirm the correctness of mutants. Double, quadruple, and hepta mutants were constructed from single mutants by the same procedure.

Construction of complementation plasmids pAhpCF and pKatA2

The 2551-bp PCR amplicon containing intact *ahpCF* genes was obtained by PCR using the primer sets AhpCF-F and AhpCF-R and cloned into pRK415, yielding pAhpCF. An approximate 2.1-kb DNA fragment containing intact *katA2* gene was obtained by PCR using primer sets KatA2N-F and KatA2C-R and cloned into pRK415, generating plasmid pKatA2.

Dihydrochodamine 123 (DHR123) assay

Overnight cultures were subcultured to fresh LB medium containing 0.9 μg/ml DHR123 with an initial OD_{450} of 0.15. After a 5-h and 24-h incubations, fluorescence was detected using 500 nm as the excitation wavelength and 550 nm as the emission wavelength.

Reverse transcription-PCR (RT-PCR)

The DNA-free RNA of logarithmic-phase *S. maltophilia* cells was extracted using Total RNA Extraction Kit Mini (ARROWTEC) and reverse transcribed to cDNA by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA of 100 ng was used as template for PCR with the primers indicated. The primer sets used were KatA1Q-F/R for *katA1*, KatA2Q-F/R for *katA2*,

KatMnQ-F/R for *katMn*, KatEQ-F/R for *katE*, AhpCQ-F/R for *ahpC*, Gpx1Q-F/R for *gpx1*, Gpx2Q-F/R for *gpx2*, and Gpx3Q-F/R for *gpx3* (Table S1). PCR amplicons were visualized by agarose gel electrophoresis. To check the specificity of primer pairs, control PCRs were performed using the chromosome DNA as the template. Since *smeX* in *S. maltophilia* KJ is intrinsically quiescent [11], it was used as the negative control to assure RNA purity.

Real time quantitative PCR (qRT-PCR)

The cDNA prepared for the aforementioned RT-PCR assay was used as template for qRT-PCR. qRT-PCR was carried out by the ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocols. The 16 s rRNA gene was used as an internal control and the transcripts of genes assayed were normalized with the internal control using $\Delta\Delta C_T$ method [12]. Primers used for qRT-PCR were the same as those used for RT-PCR (Table S1). All experiments were performed in triplicate.

Construction of promoter-*xylE* transcriptional fusion reporter plasmids

Three DNA segments upstream and including the start codons of *gpx3*, *katA2*, and *ahpC* were amplified by PCR using the primer sets of Gpx3N-F/Gpx3N-R, KatA2N-F/KatA2N-R, and AhpCN-F/AhpCN-R, respectively (Table S1). These PCR products were inserted into pRKxylE to place the amplicons upstream of *xylE*, which encodes an enzyme with C23O activity. These plasmids were referred to as $pGpx3_{xylE}$, $pKatA2_{xylE}$, and $pAhpC_{xylE}$, respectively.

Determination of C23O activity

Catechol 2, 3-dioxygenase (C23O), encoded by a *xylE* gene, catalyzes the hydrolysis of catechol into the yellow 2-hydroxymuconate semialdehyde, which can be quantitatively determined by spectrophotometric analysis at a wavelength of 375 nm. C23O activity were determined spectrophotometrically at 375 nm as described previously [11]. The rate of hydrolysis was calculated by using 44,000 M^{-1} cm^{-1} as the extinction coefficient. One unit of enzyme activity (U) was defined as the amount of C23O that converts 1 nmole catechol per min. The C23O specific activity was expressed as U/OD_{450nm}.

Growth kinetic assay

Overnight-cultured strain tested was inoculated into fresh LB medium at the initial OD_{450nm} of 0.15. The OD_{450nm} readings were taken at interval of 3 h for a total time of 24 h.

H_2O_2 susceptibility test (disk diffusion assay)

The strain tested was cultured to mid-log phase and adjusted to a concentration of 10^7 cells/ml. A 100 μl aliquot was spread evenly over the surface of a LB agar plates. A 10 μl of 20% H_2O_2 was spotted onto a sterile paper disk (6 mm in diameter) and the disk was placed on the center of plate. The diameter of growth inhibition zone around disk was measured after a 24-h incubation at 37 °C.

Results

Analysis of Kat, AhpC, and Gpx systems in *S. maltophilia* genome

The catalase (Kat), alkyl hydroperoxidase/alkyl hydroperoxide reductase (AhpCF), and glutathione peroxidase (Gpx) systems are three major and extensively reported enzymatic H_2O_2 elimination systems in several bacteria. Genome sequence analysis showed that four *kat*, one *ahpCF*, and three *gpx* genes existed in the genome of *S. maltophilia* K279a [9]: Smlt0372 (*katA1*), Smlt1385 (*katA2*), Smlt2537 (*katMn*), Smlt3583 (*katE),* Smlt0841–0840 (*ahpCF*), Smlt3183 (*gpx1*), Smlt3228 (*gpx2*), and Smlt4676 (*gpx3*). In this study, we aimed to assess the roles of the eight enzymes in alleviating hydrogen peroxide stress generated by endogenous aerobic metabolism or by exogenous sources.

AhpCF and KatA2 contribute to scavenge micromolar H_2O_2, and AhpCF play a critical role for stationary-phase cells

The intrinsic expression of the H_2O_2 scavenging enzyme genes was tested using reverse transcription-PCR (RT-PCR). Of the eight genes tested, *gpx3*, *katA2,* and *ahpC* transcripts were detected (Fig. 1a), suggesting that Gpx3, KatA2, and AhpCF may participate in the alleviation of endogenous H_2O_2 stress arising from bacterial aerobic metabolism. The expressions of *gpx3*, *katA2*, and *ahpC* genes in the logarithmic and stationary phases were further assessed by qRT-PCR. The *ahpC* expression was abundant compared to *katA2* and *gpx3* in logarithmic phase. The expression level of *ahpC* was further increased in the stationary phase (Fig. 1b). These observations suggested a critical role for *ahpC* in endogenous H_2O_2 stress alleviation.

OxyR is a well-known regulator response to H_2O_2 stress in several bacteria [13]. The regulatory role of OxyR in the intrinsic expression of *gpx3*, *katA2* and *ahpC* was assessed by qRT-PCR. The expression of *gpx3* was little affected by OxyR. The *katA2* transcript was obviously decreased in the *oxyR* null mutant, indicating that OxyR is a positive regulator for the intrinsic expression of *katA2*. Nevertheless, OxyR acted as a repressor for the expression of *ahpC* in aerobically-grown cells (Fig. 1c). This observation is peculiar since OxyR is a

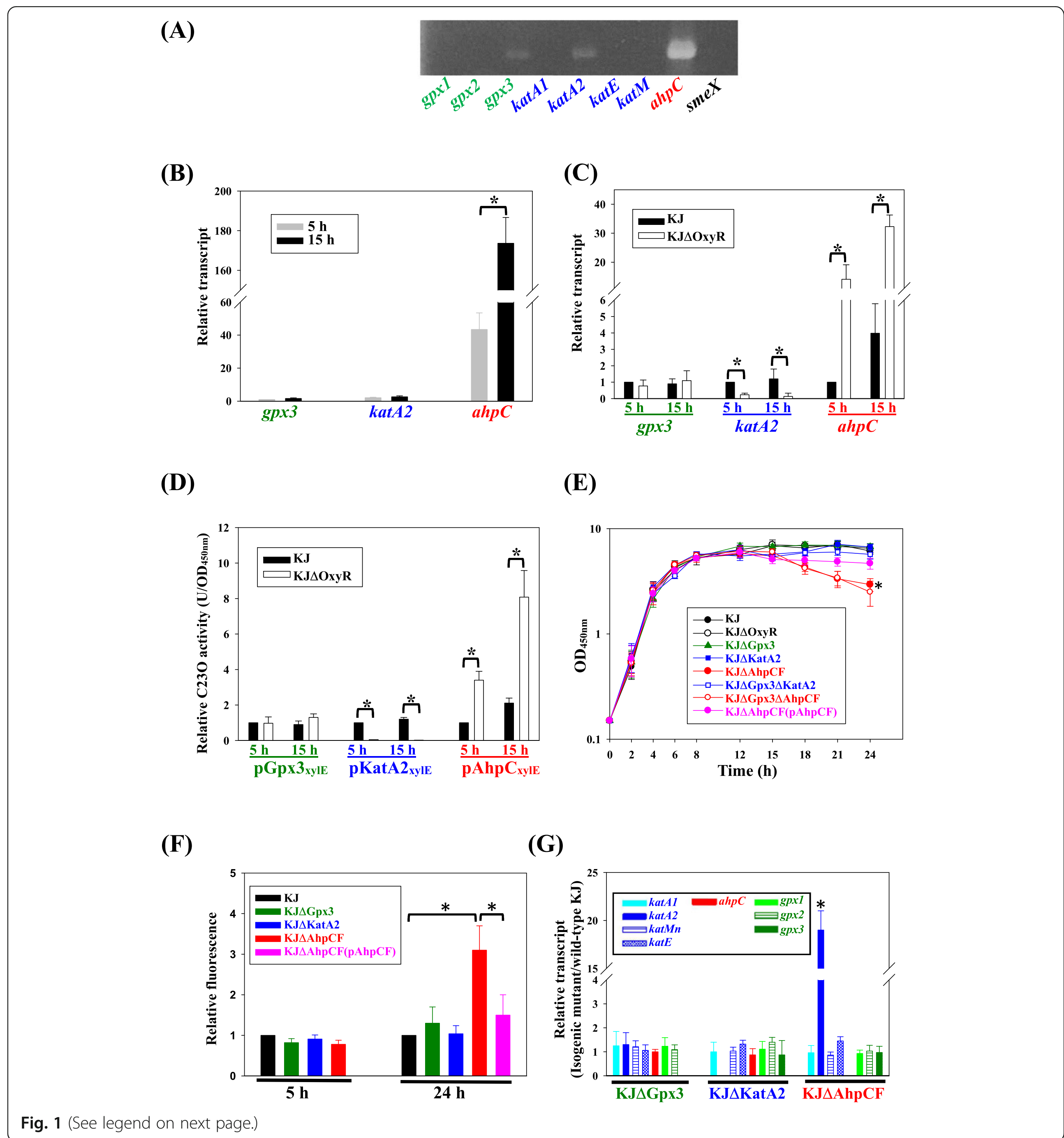

Fig. 1 (See legend on next page.)

positive regulator of antioxidant system widely reported in several bacteria [13, 14]; thus we used promoter-*xylE* transcriptional fusion construct to recheck the role of OxyR in the expression of *gpx3*, *katA2*, and *ahpC*. The same conclusion was obtained from the results of promoter-*xylE* transcriptional fusion assay (Fig. 1d). To investigate the roles of *gpx3*, *katA2*, and *ahpCF* in the alleviation of endogenously aerobic metabolism-derived H_2O_2 stress, we investigated the aerobic growth of different single mutants (KJΔGpx3, KJΔKatA2, and KJΔAhpCF) and different combinations of double mutants (KJΔGpx3ΔKatA2 and KJΔGpx3ΔAhpCF). After several tries, we could not successfully obtain the double mutant of *katA2* and *aphCF* genes. In addition, KJΔOxyR was also included. None of the tested mutants showed any observable growth restrictions in the logarithmic phase. However, *ahpCF*-associated mutants (KJΔAhpCF and KJΔGpx3ΔAhpCF) exhibited gradual

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Fig. 1 Roles of four catalases (KatA1, KatA2, KatE, and KatMn), one alkyl hydroperoxidase (AhpC), and three glutathione peroxidases (Gpx1, Gpx2, and Gpx3) in the alleviation of endogenous hydrogen peroxide stress. Bars represent the average values from three independent experiments. Error bars represent the standard error of the mean. *, $P < 0.001$, significance calculated by Student's *t* test. (**a**) Agarose gel electrophoresis of reverse transcription PCR (RT-PCR). Overnight-cultured *S. maltophilia* KJ was inoculated into fresh LB with an initial OD_{450nm} of 0.15 and grown for 5 h. The cDNAs were obtained using reverse transcription with random primers and PCR was performed using primer pairs (Table S1) targeting candidate genes. The *smeX* gene, which is not expressed in strain KJ, is used as a control for DNA contamination during cDNA preparation. (**b**) The expression of *gpx3*, *katA2*, and *ahpC* genes in logarithmic- and stationary-phase wild-type KJ cells. Overnight culture of KJ cells was inoculated into fresh LB with an initial OD_{450nm} of 0.15. Cells were grown aerobically for 5 h or 15 h before measuring *gpx3, katA2,* and *ahpC* transcripts using qRT-PCR. All values were normalized to *gpx3* transcript of logarithmic-phase KJ cells. (**c**) Regulatory role of OxyR in the intrinsic expression levels of *gpx3*, *katA2*, and *ahpC* genes. Overnight cultures of KJ and KJΔOxyR cells were inoculated into fresh LB with an initial OD_{450nm} of 0.15. Cells were grown aerobically for 5 h or 15 h before measuring *gpx3, katA2,* and *ahpC* transcripts using qRT-PCR. All values were normalized to the transcript of logarithmic-phase KJ cells. (**d**) Regulatory role of OxyR in the intrinsic expression levels of *gpx3*, *katA2*, and *ahpC* genes. Overnight cultures of bacteria cells (KJ ($Gpx3_{xylE}$), KJ ($pKatA2_{xylE}$), KJ ($pAhpC_{xylE}$), KJΔOxyR ($Gpx3_{xylE}$), KJΔOxyR ($pKatA2_{xylE}$), and KJΔOxyR ($pAhpC_{xylE}$)) were inoculated into fresh LB with an initial OD_{450nm} of 0.15. Cells were grown aerobically for 5 h or 15 h before measuring the C23O activity. All values were normalized to the activity in KJ cells. (**e**) Functions of OxyR, Gpx, Kat, and AhpCF systems in response to endogenously aerobic metabolism-derived H_2O_2 stress. The growth curves of KJ and its derived isogenic mutants were measured by reading OD_{450} at the time points as indicated. *, the growth difference of KJΔAhpCF and KJΔAhpCF (pAhpCF) at the 24-h time point was significant. (**f**) DHR 123 assay of wild-type KJ and mutants KJΔGpx3, KJΔKatA2, and KJΔAhpCF. The bacterial cells tested were cultured in LB medium containing DHR 123 for 5 h and 24 h, respectively, and the fluorescence at 550 nm was determined. The relative fluorescence is normalized to the fluorescence of wild-type KJ. (**g**) The expression levels of *gpxs*, *kats*, and *ahpCF* of KJΔGpx3, KJΔKatA2, and KJΔAhpCF in response to endogenously aerobic metabolism-derived H_2O_2 stress. Bacteria cultured overnight (KJ, KJΔGpx3, KJΔKatA2, and KJΔAhpC) were inoculated into fresh LB with an initial OD_{450nm} of 0.15 and grown for 5 h. The *katA1, katA2, katMn, katE, ahpC, gpx1, gpx2, and gpx3* transcripts were measured using qRT-PCR. The relative transcription level for each gene was expressed as the ratio of mutant to wild-type

reduction of cell density in the stationary phase, and this compromise was not observed when *ahpCF* genes were complemented (Fig. 1e).

To assess the relatedness of deletion mutant phenotypes to the intracellular H_2O_2 concentrations, the intracellular H_2O_2 concentrations of wild-type KJ and mutants KJΔGpx3, KJΔKatA2, and KJΔAhpCF in the logarithmic (5 h) and the stationary phases (24 h) were determined by dihydrochodamine 123 (DHR123) assay. DHR123 is used for the detection of intracellular ROS and can detect H_2O_2 in the presence of endogenous peroxidases. The presence of ROS oxidizes DHR123 to the fluorescent derivative rhodamine 123. Thus, the intracellular H_2O_2 concentration is proportional to the fluorescence intensity. The fluorescences detected from the logarithmic-phase KJΔGpx3, KJΔKatA2, and KJΔAhpCF, and from the stationary-phase KJΔGpx3 and KJΔKatA2 were comparable to that from wild-type KJ (Fig. 1f). Nevertheless, stationary-phase KJΔAhpCF cells had higher fluorescence relative to stationary-phase KJ cells (Fig. 1f), correlated well with stationary-phase growth compromise of *ahpCF*-associated mutants (Fig. 1e).

Given functional redundancy in these H_2O_2-alleviating enzymes, we considered the possibility that some of these enzymes may be induced to compensate for the absence of one. To test this hypothesis, the transcription levels of the eight genes were measured using qRT-PCR in the deletion mutants KJΔGpx3, KJΔKatA2, and KJΔAhpCF. Inactivation of *gpx3* or *katA2* alone did not significantly affect the expression of the other seven genes. However, the expression of *katA2* in KJΔAhpCF cells increased by 19 ± 2-fold compared to parental KJ cells (Fig. 1g).

KatA2 and AhpCF, mainly KatA2, contribute to scavenge millimolar H_2O_2

The impact of exogenous H_2O_2 stress on the expression of H_2O_2 scavenging enzymes was investigated by qRT-PCR. Of the eight genes assessed, *katA2* and *ahpC* were upregulated after a 2 mM H_2O_2 challenge (Fig. 2a).

We also assessed the possibility of compensatory expression in KJΔKatA2 and KJΔAhpCF in the presence of exogenous H_2O_2 stress. In either KJΔKatA2 or KJΔAhpCF, the expression levels of the remaining seven H_2O_2 scavenging enzymes in response to H_2O_2 challenge were hardly affected compared to that in wild-type KJ (Fig. 2a).

We investigated the regulatory role of OxyR in exogenous H_2O_2-mediated *katA2* and *ahpC* upregulation with the H_2O_2 concentrations ranged from 0 to 2 mM. When the exogenous H_2O_2 concentration was as low as 1 μM, there was no impact on the amounts of *katA2* and *ahpC* transcripts. In response to 5 μM or 100 μM H_2O_2 challenge, *katA2* transcript had a mild (approximately 2–3 fold), but not significant increment; however, *ahpC* transcript was upregulated (Fig. 2b & c). When the challenged H_2O_2 concentration was higher than 250 μM, the *katA2* and *ahpC* transcripts were significantly increased (Fig. 2b & c). In addition, *katA2* expression was positively regulated by OxyR without or with the treatment of H_2O_2 (Fig. 2b). However, OxyR regulatory role in *ahpC* expression was H_2O_2 concentration dependent, as a repressor when H_2O_2 concentration was less than 5 μM and as an activator when H_2O_2 concentration was higher than 100 μM (Fig. 2c).

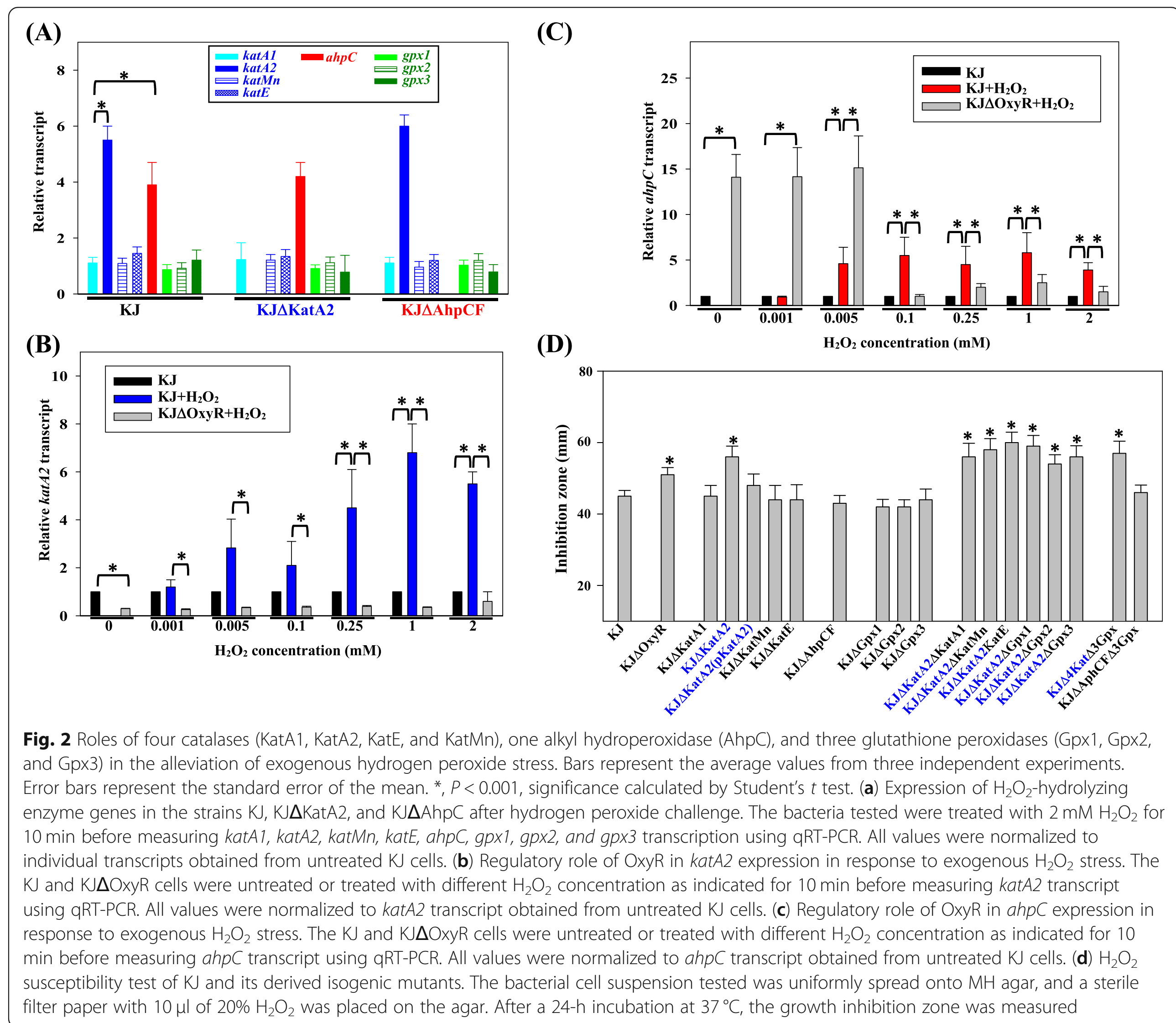

Fig. 2 Roles of four catalases (KatA1, KatA2, KatE, and KatMn), one alkyl hydroperoxidase (AhpC), and three glutathione peroxidases (Gpx1, Gpx2, and Gpx3) in the alleviation of exogenous hydrogen peroxide stress. Bars represent the average values from three independent experiments. Error bars represent the standard error of the mean. *, $P < 0.001$, significance calculated by Student's *t* test. (**a**) Expression of H_2O_2-hydrolyzing enzyme genes in the strains KJ, KJΔKatA2, and KJΔAhpC after hydrogen peroxide challenge. The bacteria tested were treated with 2 mM H_2O_2 for 10 min before measuring *katA1, katA2, katMn, katE, ahpC, gpx1, gpx2, and gpx3* transcription using qRT-PCR. All values were normalized to individual transcripts obtained from untreated KJ cells. (**b**) Regulatory role of OxyR in *katA2* expression in response to exogenous H_2O_2 stress. The KJ and KJΔOxyR cells were untreated or treated with different H_2O_2 concentration as indicated for 10 min before measuring *katA2* transcript using qRT-PCR. All values were normalized to *katA2* transcript obtained from untreated KJ cells. (**c**) Regulatory role of OxyR in *ahpC* expression in response to exogenous H_2O_2 stress. The KJ and KJΔOxyR cells were untreated or treated with different H_2O_2 concentration as indicated for 10 min before measuring *ahpC* transcript using qRT-PCR. All values were normalized to *ahpC* transcript obtained from untreated KJ cells. (**d**) H_2O_2 susceptibility test of KJ and its derived isogenic mutants. The bacterial cell suspension tested was uniformly spread onto MH agar, and a sterile filter paper with 10 μl of 20% H_2O_2 was placed on the agar. After a 24-h incubation at 37 °C, the growth inhibition zone was measured

To investigate the role of the eight enzymes in exogenous H_2O_2 detoxification, we performed an H_2O_2 susceptibility test of KJ-derived mutants containing single deletions of the *katA1*, *katA2*, *katMn*, *katE*, *ahpCF*, *gpx1*, *gpx2*, and *gpx3* genes. In addition, we assessed the H_2O_2 susceptibility of KJΔOxyR. Except for KJΔKatA2 and KJΔOxyR, the remaining seven mutants displayed H_2O_2 susceptibility that was similar to wild-type KJ (Fig. 2d). KJΔKatA2 was more sensitive to H_2O_2 than wild-type KJ (Fig. 2d), and complementation of the mutant with pKatA2, a plasmid containing an intact *katA2* gene, restored H_2O_2 resistance (Fig. 2d). KJΔOxyR was also more sensitive to H_2O_2 than wild-type KJ, but not as severe as KJΔKatA2 (Fig. 2d). Next, we assessed whether additional mutations in KJΔKatA2 could enhance H_2O_2 sensitivity by constructing several combinations of multiple genes deletion mutants using KJΔKatA2 as a parental strain and performing H_2O_2 sensitivity assays in all mutants. H_2O_2 sensitivity was hardly augmented compared to KJΔKatA2 in all mutants tested, although 4 catalase genes and three *gpx* genes were simultaneously inactivated (KJΔ4KatΔ3Gpx) (Fig. 2d).

It has been reported that OxyR of *E. coli* binds to the 5′ promoter-operator regions of target genes at a conserved motif comprised of four ATAG elements spaced at 10-bp intervals [15, 16]. Since OxyR is involved in the H_2O_2-induced upregulation of *katA2* and *ahpCF*, we surveyed the upstream region of the *ahpCF* and *katA2* genes. We discovered ATAG-N14-ATAG and ATAG-N19-ATAG elements near the *ahpCF* and *katA2* promoters (Fig. S1).

Discussion

H_2O_2 stress is an inevitable challenge for aerobic bacteria. Respiratory bursts account for up to 87% of the

total H_2O_2 production in aerobically-grown *Escherichia coli,* and intracellular H_2O_2 from aerobic metabolism normally remains at low-micromolar ranges (< 4 μM) [17]. In the course of infection, H_2O_2 levels can reach up to millimolar concentrations because of the oxidative burst generated by host immune cells [2]. To avoid H_2O_2 toxicity, bacteria have equipped themselves with several scavenging enzymes to maintain intracellular H_2O_2 at nanomolar concentrations [4, 17]. AhpCF and catalase systems are scavenging enzymes that are extensively conserved in several bacterial lineages [2]. AhpCF is more kinetically efficient than catalases at scavenging H_2O_2, but its activity is more easily saturated than that of catalases [4]. Therefore, AhpCF is the primary scavenger when H_2O_2 is in the low-micromolar range, and catalase activity predominates when the cell reaches millimolar levels of H_2O_2 [4]. This paradigm has been observed in a variety of organisms [4], and we highlight our findings in this study to add new evidence to this paradigm.

AhpCF of *S. maltophilia* was expressed in the logarithmic phase and further upregulated in the stationary phase (Fig. 1b), implying that higher AhpCF activity is required for *S. maltophilia* to deal with H_2O_2 stress in the stationary phase. This inference is supported by the observation in Fig. 1e and Fig. 1f, since *ahpCF*-associated mutants (KJΔAhpCF and KJΔGpx3ΔAhpCF) exhibited compromised stationary-phase growth (Fig. 1e) and the higher H_2O_2 concentration was observed in the stationary-phase KJΔAhpCF cells (Fig. 1f). Inactivation of *katA2* did not affect the expression of other H_2O_2 scavenging enzymes (Fig. 1g) and did not compromise bacterial aerobic growth (Fig. 1e), indicating that AhpCF alone is potent enough to deal with the low-micromolar H_2O_2 stress. In contrast, upregulation of KatA2 is needed to attain normal logarithmic growth in the case of *ahpCF* inactivation (KJΔAhpCF) (Fig. 1e and g). Collectively, for an aerobically-grown *S. maltophilia*, AhpCF and KatA2 are key enzymes responsible for the alleviation of logarithmic-phase H_2O_2 stress and AhpCF system plays a critical role in dealing with the stationary-phase H_2O_2 stress.

When bacteria encounter exogenous H_2O_2 stress up to the high-micromolar, even millimolar level, *ahpCF* and *katA2* are upregulated (Fig. 2a), linking the contribution of AhpCF and KatA2 to alleviate high concentration H_2O_2. However, neither KJΔKatA2 nor KJΔAhpCF exhibited compensatory expression of other enzymes tested in response to 2 mM H_2O_2 challenge (Fig. 2a), suggesting that there should be other non-enzymatic systems contributing to deal with millimolar H_2O_2 stress in addition to KatA2 and AhpCF. However, we also observed that the *katA2*-associated mutants, but not the other mutants, had a compromised H_2O_2 tolerance (Fig. 2d), indicating that among the enzymes tested in this study, KatA2 is the dominant enzyme for the alleviation of high concentration H_2O_2 stress.

Vattanaviboon's group has investigated the role of AhpCF of *S. maltophilia* in response to high level of H_2O_2 stress recently [18], and their conclusions are not totally consistent with our findings. They demonstrated that inactivation of *ahpC* rendered *S. maltophilia* more resistant to 300–900 mM H_2O_2 than parental strain, which was attributed to the enhanced KatA2 expression and activity [18]. However, our results showed that the expression of *katA2* in the 2 mM H_2O_2-treated *ahpCF* mutant (KJΔAhpCF) was comparable to that of parental strain (KJ) (Fig. 2a). The discrepancy may be attributed to different stress intensities (the treated H_2O_2 concentration and time intervals), different experiment designs for H_2O_2 tolerance evaluation, and strain variation. If the *ahpC* mutant indeed gains a survival superiority against H_2O_2 at concentrations commonly used in a hospital, the prevalence of *ahpC* mutant in the clinical *S. maltophilia* isolates should be an interesting issue to study.

The OxyR regulatory role is another interesting finding in this study. OxyR is an H_2O_2-sensing transcriptional regulator that is generally conserved in Gram-negative bacteria [13, 14]. In this study, H_2O_2 dose-dependent regulation was observed in *S. maltophilia* OxyR. OxyR functioned as a positive regulator for the expression of *katA2* either at micromolar or at millimolar H_2O_2 concentrations (Fig. 1c, d, & b). However, OxyR played a double-edged role in the regulation of *ahpCF* expression. OxyR repressed *ahpCF* expression at low-micromolar H_2O_2 concentrations (H_2O_2 concentration < 5 μM) (Fig. 1c, d & c), but activated *ahpCF* expression when H_2O_2 H_2O_2 concentration higher than 100 μM (Fig. 2c). This is uncommon because OxyR generally promotes *ahpCF* expression in other bacteria [19]. Herein, we proposed two possibilities to explain this observation. (i) Two different OxyR activated forms may form dependent on the H_2O_2 concentrations (different symbols for active OxyR in Fig. 3a and b), which may have different impacts on *ahpCF* expression (Fig. 3). (ii) Members of OxyR regulon triggered by low H_2O_2 concentrations are not totally the same as those triggered by high H_2O_2 concentrations, and different OxyR regulon member(s) regulate(s) the *ahpCF* expression in micromolar and millimolar H_2O_2 concentrations, respectively (Fig. 3a and b). The negative regulatory role of OxyR in *ahpCF* expression (Fig. 1c, d) may help *S. maltophilia* to cope with the endogenous H_2O_2 stress in the case of the loss of OxyR function. When *oxyR*

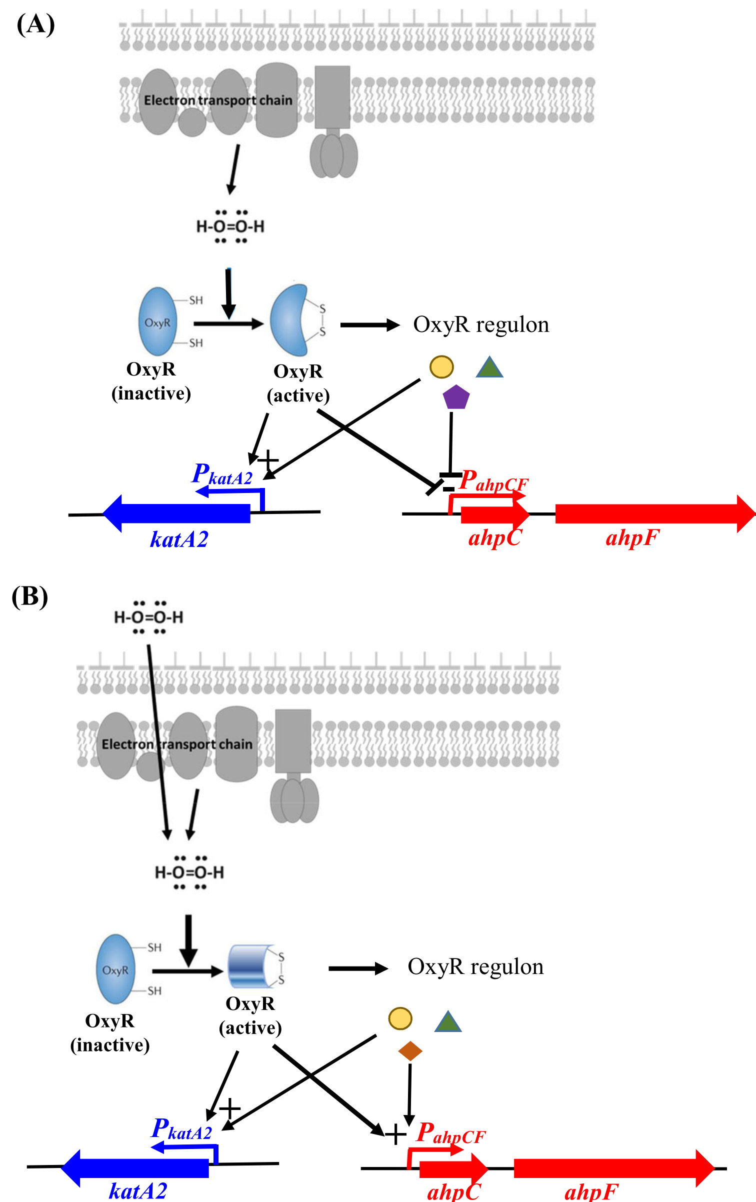

Fig. 3 A model for H_2O_2-dependent and OxyR-mediated transcription regulation of *ahpCF* and *katA2* genes in response to different concentrations of H_2O_2 stress in *S. maltophilia*. (**a**) Low-micromolar H_2O_2 is generated by bacterial aerobic metabolism and OxyR is oxidized at a specific "sensing" cysteine residue by H_2O_2. The activated OxyR represses the expression of *ahpCF* operon and increases the expression of *katA2* gene, either directly or indirectly. (**b**) When bacteria encounter exogenous H_2O_2 stress and the intracellular H_2O_2 concentration increases to millimolar levels, activated OxyR activates the expression of *ahpCF* operon and *katA2* gene, either directly or indirectly

is inactivated, the shortage of KatA2 activity can be compensated by upregulated AhpCF, which can maintain normal H_2O_2 detoxification. This may be the reason why KJΔOxyR displayed comparable growth with wild-type KJ, but KJΔAhpCF had a growth compromise in the stationary phase (Fig. 1e).

Conclusion

AhpCF and KatA2 are two main enzymes to differentially protect *S. maltophilia* from the hydrogen peroxide stress. AhpCF and KatA2 participate the alleviation of low-micromolar level H_2O_2, and AhpCF has a crucial role for stationary-phase cells; in contrast, KatA2 is the major contributor for dealing with the millimolar level H_2O_2. OxyR acts as a positive regulator for the expression of *katA2*. However, the regulatory role of OxyR in the *ahpCF* expression depends on the H_2O_2 concentration, as a repressor in H_2O_2 of low-micromolar level and as an activator in H_2O_2 of millimolar level.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12929-020-00631-4.

Additional file 1 Table S1. Bacterial strains, plasmids and primers used in this study.

Additional file 2 Fig. S1. Analysis of putative OxyR binding motifs in the upstream regions of *katA2* and *ahpC* genes.

Abbreviations

Ahp: Alkyl hydroperoxidase; DHR123: Dihydrochodamine 123; Gpx: Glutathione peroxidase; Kat: Catalase; QRT-PCR: Real time quantitative PCR; ROS: Reactive oxygen species; RT-PCR: Reverse transcription-PCR

Acknowledgements

Not applicable.

Author's contributions

LHL and YLS conceived the experiment and analyzed data. JYH, CJW, YWH, HHH, and YCT performed the experiments, generated and collected the data. TCY designed the experiment, interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

Funding

This study was funded by grant MOST 108–2320-B-010-032-MY3 from the Ministry of Science and Technology of Taiwan, grants V108B-037 and V109C-195 from Taipei Veterans General Hospital, and grant 2019SKHAND007 from the Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan.

Availability of data and materials

Data and materials related to this study are available upon request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 25 July 2019 Accepted: 17 February 2020
Published online: 25 February 2020

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