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Role of *Burkholderia pseudomallei* RpoS in Regulation of Catalase Activities under Hydrogen Peroxide Induction

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ABSTRACT: *Burkholderia pseudomallei* is the causative agent of melioidosis. A previous study showed that the *B. pseudomallei rpoS* mutant is more sensitive to hydrogen peroxide than is the wild type strain, suggesting the RpoS may play a critical role in resistance to oxidative stress. Analysis of *rpoS* gene expression by β-galactosidase activity assay indicated that *rpoS* is activated when exposed to hydrogen peroxide on entry into the stationary phase. In addition, native gel electrophoresis and staining for catalase activities revealed that there are two types of catalases (I and II) in *B. pseudomallei*. Catalase I in the *rpoS* mutant exhibited higher activity than the parent strain, whereas catalase II was not detected in the *rpoS* mutant strain. Interestingly, even though catalase II was the prominent activity in the stationary phase culture of the wild type, it did not increase in response to hydrogen peroxide treatment. Construction of the *B. pseudomallei katE* mutant indicated that the *katE* gene encodes catalase II. Our experiments showed that *katE* is directly controlled by the RpoS and is not induced by hydrogen peroxide. These results suggest that the *B. pseudomallei* RpoS is activated under an oxidative stress condition and controls the activities of both catalases, I and II, in different manners.

KEYWORDS: *Burkholderia pseudomallei*, RpoS, catalase, hydrogen peroxide.

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis. *B. pseudomallei* has been found to survive and multiply in human phagocytes *in vitro* and can also persist in a dormant stage in macrophages for months or years[1]. Its ability to survive under environmental stress conditions and to be present intracellularly for a long period of time remains to be elucidated. The *rpoS* gene has been shown to be important for survival during environmental stress in several bacterial species. The sigma factor RpoS (s^S) was originally identified in *Escherichia coli* and *Salmonella typhimurium* as an alternative sigma factor that activates the expression of numerous genes required to maintain cell viability during the stationary phase of growth when cells are experiencing nutrient starvation[1,2]. Activation of these genes makes the bacterium more resistant to environmental stresses, such as prolonged starvation, osmotic stress, and oxidative stress.

The *B. pseudomallei rpoS* gene has been identified and is located downstream of an *nlpD*-like gene in the same orientation as found in many other bacterial species[3]. A *rpoS* mutant was constructed and analyzed for its response to various environmental stresses in comparison with the parental strain. The results suggested that *rpoS* plays a critical role in resistance to hydrogen peroxide in *B. pseudomallei*. In addition, superoxide dismutase and catalase enzymes significantly inhibit macrophage bactericidal activity against *B. pseudomallei in vitro* suggesting the importance of antioxidant enzymes for the intracellular survival of this pathogen[4,5].

In this study, we attempted to monitor the *B. pseudomallei rpoS* gene expression and its role in regulation of catalase activities when the cells are exposed to hydrogen peroxide. Using β-galactosidase activity assay, the *rpoS* expression was found to be significantly activated by hydrogen peroxide treatment upon the entry into the stationary phase. The zymogram study of catalase enzyme activities in native gel electrophoresis revealed 2 types of catalase enzymes (catalase I and catalase II). We also demonstrated that the *katE* gene encoding the catalase II was directly regulated by RpoS, but it was not induced by hydrogen peroxide. Moreover, the *katG* gene encoding catalase I was suggested to play an important role in oxidative stress conditions at the stationary phase, particularly

Table 1. Bacterial strains.

Strain	Genotye or relevant characteristics	Source of references
B. pseudomallei		
PP844	Prototroph, blood culture isolate from a patient at Khon Kaen University Hospital	Utaisincharoen *et al*. (2001)
KN100	PP844 *rpoS* mutant	Subsin *et al*. (2003)
Z2BS1	PP844 containing *rpoS::lacZ* transcriptional fusion	Subsin *et al*. (2003)
G221	PP844 *katG* mutant	Loprasert *et al*. (2003)
E221	PP844 *katE* mutant	This study

when the bacteria lacked RpoS.

Materials and Methods

Bacterial Strain and Growth Conditions

The bacterial strains used are listed in Table 1. *B. pseudomallei* was routinely maintained in Luria-Bertani (LB) medium[6]. *Pseudomonas* agar base supplemented with SR103E (cetrimide, fucidin, and cephaloridine) from Oxoid was used after conjugation as selective medium to inhibit growth of *E. coli*. All cultures were grown at 37°C, at 250 rpm shaking with aeration. Tetracycline (60 mg/ml), chloramphenicol (40 mg/ml) and trimethoprim (100 mg/ml) were added to media when required.

Assay for the Induction of the *rpoS* Promoter by Hydrogen Peroxide

Overnight cultures of *B. pseudomallei* Z2BS1 were sub-cultured ($O.D._{600}$~0.1) into LB at 37°C with aeration. Each stage was induced with 0.5 mM hydrogen peroxide every 10 min for 1 h before harvesting, then assayed for β-galactosidase activity[7].

Enzyme Assays

Cells carrying the transcriptional fusion constructs were grown in LB medium overnight, and 1% was inoculated into fresh LB medium. Aliquots of the cultures were taken at intervals to follow the cell growth. Each aliquot was assayed for β-galactosidase activity as described by Miller[7]. All cultures were assayed in triplicates, and reported values are averaged from at least three independent experiments.

Oxidative Stress Conditions

Oxidative stress conditions were obtained by adding 0.5 mM H_2O_2 to LB cultures. Cells at each growth condition were induced with 0.5 mM H_2O_2 every 10 min for 1 h before harvest. After the last treatment, the cells were removed and washed once with phosphate-buffer-saline (PBS) before use.

Preparation of Crude Extracts and Measurement of Protein

B. pseudomallei cells were lysed by sonication of washed cultures previously resuspended in phosphate buffer (5 mM potassium phosphate, pH 7.0, 5 mM EDTA, 10% glycerol, 25 mM phenylmethylsulfonyl fluoride) to one-tenth of the original culture volume. Care was taken to avoid destruction of heat-labile catalase activity during sonication; typically, cells were put on ice and the suspension sonicated with an ultrasonic probe at 4°C to disrupt the cells and to fragment the genomic DNA. The sonication condition was 90 second bursts, typically 3 times. Debris was pelleted by centrifugation at 12,000x g, for 10 min at 4°C. The total protein concentration in each sample was determined with Bradford Reagent (Sigma Chemical, St. Louis, MO).

Activity Staining for Catalase

Catalase I and catalase II activity present in crude extracts of *B. pseudomallei* cells was determined by loading 20 μg of protein in a 10% non-denaturing polyacrylamide gel. After polyacrylamide gel electrophoresis, the gels were washed with distilled water three times (20 min each) to remove surface-attached buffer ions and were treated with 0.003% hydrogen peroxide for 10 min. The gels were then incubated in a solution of 2% (w/v) ferric chloride-potassium ferric cyanide until the gel background was stained green[8]. Quantification of band intensities represented as a densitometric graph was done with the Quantity One software, version 4.4.0 (BIO-RAD) in the Volume Overlay Tools Mode. Values of catalase activity from samples were divided by those from the catalase I activity at 3 h growth.

Genome Search for *katE* Genes

Based on the *B. pseudomallei* strain K96243 genome project, a *katE* gene was annotated and reported in NCBI with accession number BX 571966. The amino acid sequence of a *katE*-like gene of *B. pseudomallei*

strain K96243 showed 56.9% identity to *Escherichia coli* and 62.95% identity to *Pseudomonas putida katE* genes.

Construction of a *katE* Knockout Mutant Strain

A *katE* knockout mutant was created by insertion of a chloramphenicol resistance plasmid into *katE*. Briefly, a 300-bp *Sac*II-*Sal*I fragment of the *katE* gene was ligated into a suicide vector pKNOCK-Cm[9] to create E221, which was then mobilized from *E. coli* S17-1λpir into *B. pseudomallei* by conjugation. The single-crossover occurred within the *katE* on the chromosome. Mutants were selected on *Pseudomonas* agar containing 40 mg/ml chloramphenicol. E221 was shown by Southern analysis to have the desired gene disruption.

Results

Hydrogen Peroxide-Dependent Activation of *rpoS* Expression

To determine whether hydrogen peroxide regulates *rpoS* in *B. pseudomallei*, we used a *B. pseudomallei rpoS::lacZ* reporter fusion, Z2BS1, as listed in the Materials and Methods. The activities of the reporter in Z2BS1 were compared at every stages of the bacterial growth in the presence and absence of 0.5 mM hydrogen peroxide. Analysis of the *rpoS* gene expression via β-galactosidase activity indicated that *rpoS* was activated when exposed to hydrogen peroxide, and was maximally expressed in the early stationary phase or between 6-12 h of growth (Fig. 1). After 24 h of growth, the *rpoS* gene expression was not stimulated by hydrogen peroxide.

Catalase Activity

Catalase activity in the extract of wild-type *B. pseudomallei* strain PP844 was present in a time-dependent manner during the bacterial growth in rich medium, as seen by comparing the native gel electrophoresis zymograms shown in Fig. 2A. In the *B. pseudomallei* wild-type strain, two types of catalase activities were found, catalase I and catalase II. Catalase I activity was observed in every stage of the growth with slightly increased activity from 10 to 24 h, followed by a plateau. Catalase II activity was detected at the late stationary phase (after 48 h of growth). In late stationary phase (72 to 96 h), catalase I activity decreased, but catalase II activity showed an induction as shown in Figs. 2A and 2a.

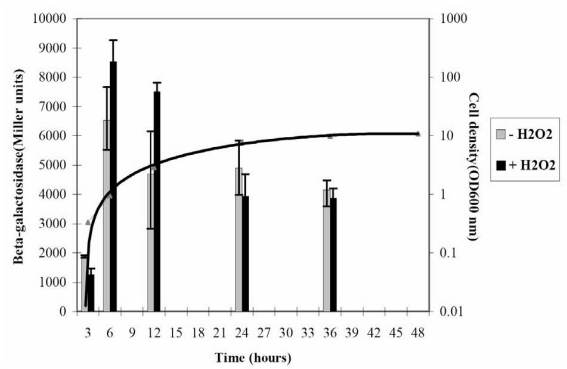

Fig 1. Expression of the *rpoS* promoter in response to 0.5 mM hydrogrn peroxide. β-galactosidase activities in crude extracts of the *rpoS::lacZ* fusion prepared from uninduced cells and H_2O_2-induced cells in various stages.

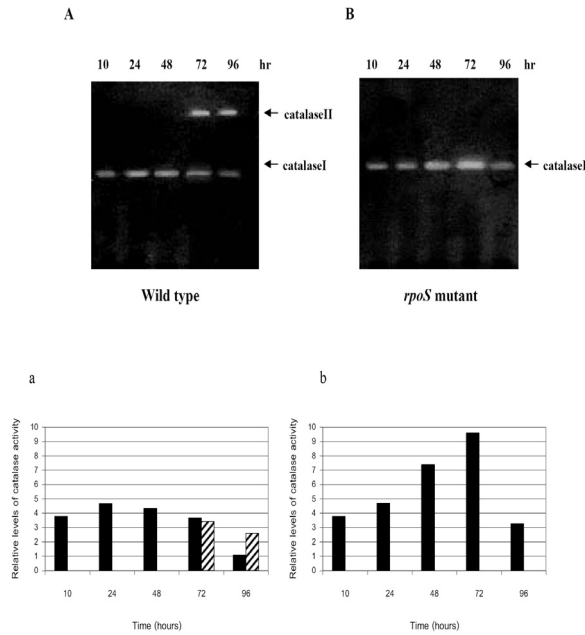

Fig 2. Zymography of catalase activity in various stages. **(A)** *B. pseudomallei* PP844 was grown aerobically in LB medium, and cell extracts (15μg) were prepared as described in the Materials and Methods. The extracts were electrophoresed in 10% nondenaturing polyacrylamide gel, and stained for catalase activity. **(B)** Crude extracts (15μg) prepared from the *rpoS* mutant. Lanes: 1-5 are at 10, 24, 48, 72, and 96 h, respectively. Densitometric quantification (**a** and **b** correspond to **A** and **B** above, respectively) of catalase activity using the Quantity One software (BIO-RAD), black bars represent data from catalase I activity and hatched bars from catalase II activity.

Catalase Activity in the *rpoS* Mutants

To determine whether a mutation in the *rpoS* gene would affect catalase activity, the KN100 strain was used. Whole-cell extracts of both wild-type and *rpoS* mutant strains were obtained from cultures at several time points. Catalase activities were assayed on non-denaturing acrylamide gels, as shown in Fig. 2B. In the

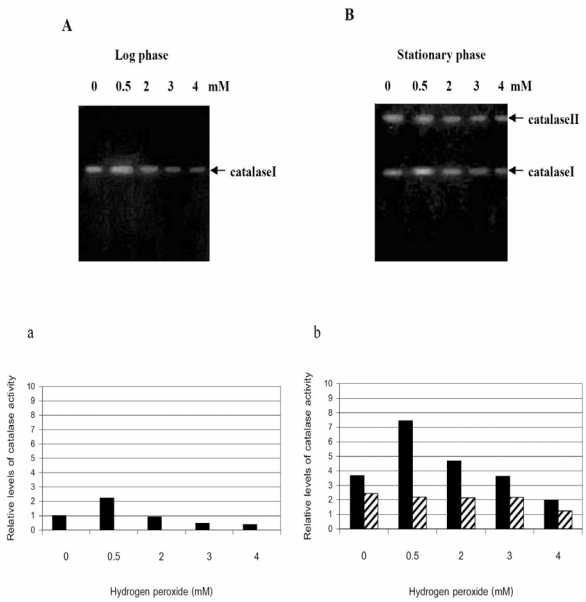

Fig 3. Non-denaturing gels showing activity staining of catalases in bacterial crude extracts. (**A**) Crude extracts (15 μg) prepared from the wild type strain in the exponential phase (3 h) which had been treated with 0, 0.5, 2, 3, 4 mM hydrogen peroxide every 10 min for 1 h. (**B**) Crude extracts (15 μg) prepared from the wild type strain in the late stationary phase (60 h) which had been treated with 0, 0.5, 2, 3, 4 mM hydrogen peroxide every 10 min for 1 h. Densitometric quantification (**a** and **b** are corresponding to **A** and **B** above) of catalase activity using the Quantity One software (BIO-RAD), black bars represent data from catalase I activity and hatched bars from catalase II activity.

rpoS mutant, the intensity of catalase I was significantly increased at 48 and 72 h of growth in comparison to the wild-type (Fig. 2b). Strikingly, catalase II activity was undetectable in the *rpoS* mutant.

Dose-Response of Hydrogen Peroxide on the Expression of Catalase I and Catalase II

To obtain dose-response activities of catalases towards an oxidative stress substance, hydrogen peroxide, the mid-exponential-phase and the stationary phase cultures of the wild-type *B. pseudomallei* were periodically exposed to various concentrations of hydrogen peroxide (from 0.5 mM to 4 mM) every 10 min for 1 h before harvest, and the catalase activities were determined. As shown in Fig. 3, the strongest induction of catalase I in the mid-exponential-phase culture by hydrogen peroxide was at 0.5 mM, whereas at the concentration of 4 mM, hydrogen peroxide became lethal. In the stationary phase culture, catalase I, but not II, can be induced by 0.5 mM hydrogen peroxide.

Effect of Hydrogen Peroxide on Catalase I and II During Growth

Considering the importance of catalase in oxidative stress, it was of interest to compare the catalase activities between *B. pseudomallei* wild-type and the *rpoS* mutant strains under hydrogen peroxide induction. Fig. 4 showed the catalase I and catalase II activities at the mid-log phase (3 h), the early stationary phase (12 h) and the late stationary phase (60 h) from both strains.

In general, the wild-type and the *rpoS* mutant strains had low levels of catalase I and catalase II activities. However, the treatment of 0.5 mM hydrogen peroxide slightly affected the catalase I activity of the wild-type, whereas catalase II activity was still undetectable.

In cultures grown to the early stationary phase, after the induction with hydrogen peroxide as previously described, catalase I activity increased in the wild-type strain but to a lesser extent than in the cultures grown to the mid-log phase (Fig. 4). The level of catalase I activity in the *rpoS* mutant was higher than that in the wild-type, as shown in Fig. 2. Moreover, the catalase I activity of the mutant was not induced by the treatment with hydrogen peroxide. The catalase II activity was not detected (Fig. 4).

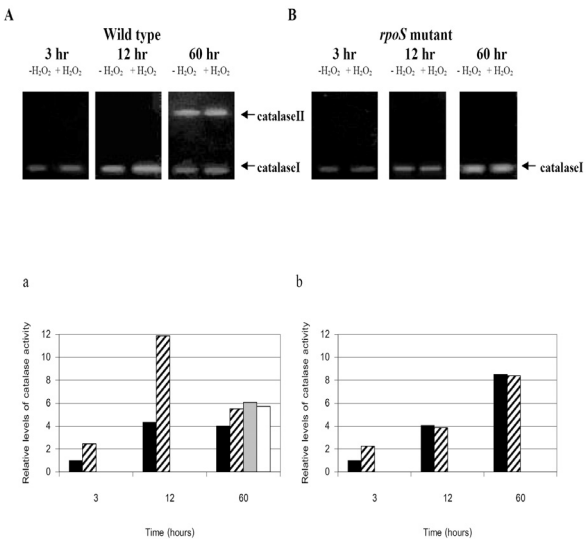

Fig 4. Zymograms of non-denaturing gels showing catalase activities in bacterial crude extracts. **(A)** Crude extracts (15 μg) prepared from the wild-type at various stages which had been treated with either 0 or 0.5 mM Hydrogen peroxide every 10 min for 1 h. **(B)** Crude extracts (15 μg) prepared from the *rpoS* mutant at various stages which had been treated with either 0 or 0.5 mM hydrogen peroxide every 10 min for 1 h. Densitometric quantification (**a** and **b** correspond to **A** and **B** above, respectively) of catalase activity using the Quantity One software (BIO-RAD), black and gray bars represent catalase I and II activities, respectively; hatched and open bars represent catalase I and II from cultured treated with hydrogen peroxide, respectively.

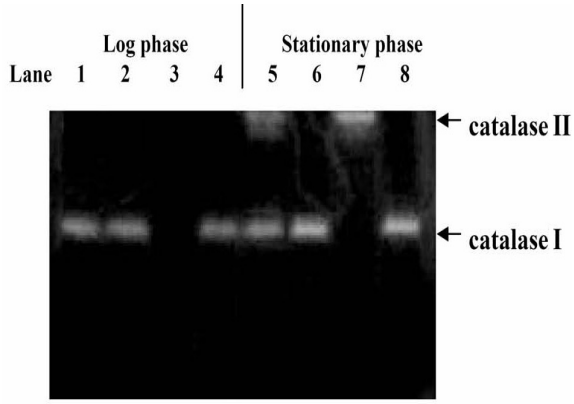

Fig 5. Zymograms of non-denaturing gels showing catalase activities in bacterial crude extracts. Lanes: 1, wild-type at log phase; 2, *rpoS* mutant at log phase; 3, *katG* mutant at log phase; 4, *katE* mutant at log phase; 5, wild-type at stationary phase; 6, *rpoS* mutant at stationary phase; 7, *katG* mutant at stationary phase; 8, *katE* mutant at stationary phase.

In the late stationary phase, both catalase I and catalase II activities were detected in the wild-type and the catalase I activity was activated by hydrogen peroxide, whereas the catalase II activity was not (Fig. 4A). This data indicated that hydrogen peroxide was not required for the induction of catalase II activity. Catalase I and catalase II activity analyses of the *rpoS* mutant in the late stationary phase showed that the catalase I activity was not activated by hydrogen peroxide, while catalase II was still not detected (Fig. 4B).

Phenotypic Comparison of *rpoS*, *katG* and *katE* Mutants

In order to measure catalase activties in various catalase-deficient strains at the log phase and stationary phase using zymography, we determined that the lower band was an electrophoretic variant of catalase I (KatG), and the upper band was catalase II (KatE), as shown in Figure 5. We also measured the catalase I levels in a strain in which the catalase II structural gene, *katE*, had been inactivated (Fig. 5). The result showed induction of catalase I at the stationary phase similar to that of the parent strain, PP844, though *rpoS* is intact.

Discussion

It is established that *E. coli rpo*S is expressed at relatively low levels in the exponential phase but is induced when cells enter the stationary phase in rich medium[10], as also found in *B. pseudomallei*[3]. However, the expression level of *B. pseudomallei rpo*S under oxidative stress had not yet demonstrated. We, therefore, further examined the *rpoS* expression under hydrogen peroxide induction and found higher expression of *rpoS* in the early stationary phase when compared to the normal condition. Little or no change occurred during exponential growth or up to 24 h of growth. The result indicated that the role of RpoS under hydrogen peroxide treatment is active only in the early stationary growth of 6 to 12 h. Our report presents for the first time a relationship between RpoS and hydrogen peroxide induction in *B. pseudomallei*.

Catalase is a ubiquitous, well-studied enzyme that catalyses the decomposition of hydrogen peroxide into water and oxygen to protect cells from the damaging effects of hydrogen peroxide[11-13]. In this study, we reported 2 types of catalase enzyme, so-called catalase I and catalase II, in the *B. pseudomallei* (as shown in Fig.2A). Catalases I and II are encoded by the *kat*G and *kat*E genes, respectively (see Fig. 5). In *E. coli* the KatE is known to be regulated by RpoS, consequently cellular expression of this enzyme increases at the onset of the stationary phase[14,15]. From the activity gel in Fig. 2, the detected upper band most likely represented KatE, since it was absent in the *rpoS* and *katE* mutants. Interestingly, the *rpoS* mutant did not possess a catalase activity band that migrated with KatE. Furthermore, catalase assays indicated that *katE* might be directly controlled by RpoS. These results agree with published reports of a major increase in the synthesis of the RpoS-dependent HPII in *E. coli*[16].

It has been noted that the *B. pseudomallei katG* expression is induced at the transcriptional level by the OxyR regulator[17]. Moreover, a typical OxyR binding site has been found in the *B. pseudomallei katG* promoter. However the catalase I activity reported in this study was significantly increased in the *rpoS* mutant compared to the wild-type strain (Fig.2). The survival during carbon starvation of the *rpoS* mutant strain cultured in glucose minimal medium has been previously reported by Subsin *et al*.[3], who found that less than 10% of the *rpoS* mutant *B. pseudomallei* could survive after 48 h. Moreover, after 72 and 96 h of cultivation, only 1% of the bacteria survived. However, in this study we have cultured the bacteria in LB medium and found that at 72 and 96 h, the *rpoS* mutant strain showed a 31% and 29% survival rate (data not shown), indicating that cells in late stationary phase in rich media had a higher survival rate compared to those grown in minimal media. In *E. coli*, an increase of HPI catalase activity in stationary phase, attributed to RpoS-dependent transcription of *kat*G has been reported[18]. In the *rpoS* mutants, these authors[15] detected little or no catalase activities by zymography assays. In contrast, our results agree with the published reports of Visick and Clarke[21] that observed higher HPI activity in the *rpoS* mutant (~50%) than in the wild type. Our results from several assays of many different extracts indicated significant levels of catalase I, which was further confirmed by means of

activity gel zymography and by *katG* promoter fusion activity assay (data not shown).

Catalases have been shown to be important enzymes for the survival of facultative aerobic organisms exposed to hydrogen peroxide. During exponential growth, the activities of both catalase I and II in *E. coli* are low, but HPI activity is inducible by hydrogen peroxide[19]. In order to test the effects of hydrogen peroxide to induce catalase activities in *B. pseudomallei*, a dose-response effect was used. Our results suggested that the strongest induction of *B. pseudomallei* catalase activities were evoked by 0.5 mM hydrogen peroxide (Fig. 3). The catalase I and catalase II activities of the parent strain (PP844) grown 3 to 60 h revealed low level of catalase I. After 0.5 mM hydrogen peroxide was added every 10 minutes for 1 h, however, catalase I activity increased in this strain (Fig. 4). These results were consistent with previous reports[20,21]. No change in catalase II activity was observed. In the *rpoS* mutant strain (KN100), catalase I increased in the exponential phase (3 h). Similar basal levels of catalase activity were observed for the *rpoS* mutant (KN100), but neither catalase I nor catalase II was induced by hydrogen peroxide in this strain upon entry into stationary phase. It has been reported that the HPI is not inducible in the absence of a positive regulator of *katG* transcription[20].

Similar results were obtained in *E. coli*, since catalase activity was found to fluctuate markedly depending upon the phase of growth[22]. For example, *kat*G (encoding HPI) is expressed during the mid-logarithmic phase and is inducible by hydrogen peroxide, while *kat*E (encoding HPII) is expressed during the late log to stationary phase and is not inducible by hydrogen peroxide[16].

Moreover, it has been reported that KatG of *B. pseudomallei* is repressed by OxyR, and induced by hydrogen peroxide via OxyR de-repression[17]. Interestingly, we found that the *rpoS* mutant has higher catalase I activity than the wild-type in the late stationary phase. Further investigation of the correlation between OxyR and RpoS may help to elucidate the mechanism of induction of the *katG* gene.

Comparison of catalase I and catalase II activities in the different mutants (*rpoS*, *katG*, and *katE* mutants) revealed that the *katE* mutant showed an induction of catalase I at the stationary phase relative to that of the wild-type strain, PP844, although *rpoS* is intact. This result supported the hypothesis that catalase I activity increased in response to elevated oxidative stress in the catalase II-deficient cells[18]. In summary, the data presented here suggests the existence of a secondary pathway for catalase I induction at the stationary phase of *B. pseudomallei*. Since the catalase I level is elevated in the *katE* mutants, we propose that this may be a response to the increased oxidative stress in the absence of catalase II. Apparently, the increase of catalase I in the *rpoS* mutant is either due to the elevation of OxyR or to other oxidative stress response regulatory networks, which are under investigation.

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