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SHORT COMMUNICATION

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DpsA protects the human pathogen *Burkholderia pseudomallei* against organic hydroperoxide

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Abstract The human pathogen, *Burkholderia pseudomalle*, is able to survive and multiply in hostile environments such as within macrophages. In an attempt to understand its strategy to cope with oxidative stress, the physiological role and gene regulation of a nonspecific DNA-binding protein (DpsA) was investigated. Expression of *dpsA* increases in response to oxidative stress through increased transcription from the upstream *katG* (catalase–peroxidase) promoter, which is OxyR dependent. *dpsA* is also transcribed from its own promoter, which is activated by osmotic stress in an OxyR-independent manner. DpsA-deficient mutants are hypersensitive to *tert*-butyl hydroperoxide, while overexpression of DpsA leads to increased resistance to organic oxidants. *B. pseudomallei* DpsA can also protect *Escherichia coli* against organic hydroperoxide toxicity. The mechanism of DpsA-mediated resistance to organic hydroperoxides was shown to differ from that of alkyl hydroperoxide reductase.

Keywords Melioidosis · Oxidative stress · Nonspecific DNA-binding protein · Organic hydroperoxide

Introduction

Aerobic microorganisms have evolved a variety of mechanisms to protect their DNA from oxidative damage caused by reactive oxygen species generated during metabolism or present in the environment. In *Escherichia coli*, the best studied bacterium, conditions of either oxidative or nutritional stress cause the production of high levels of the nonspecific DNA-binding protein Dps, which effectively protects DNA against oxidants (Martinez and Kolter 1997). *E. coli* Dps and its homologue in *Bacillus subtilis*, MrgA, are highly abundant in stationary-phase cells, where the proteins bind DNA without apparent specificity to form extremely stable complexes (Almiron et al. 1992; Chen and Helmann 1995). Dps forms spherical dodecamers, homologous to ferritins, that sequester and protect DNA from damage due to oxidative stress, nucleases, UV light, and acid stress (Wolf et al. 1999; Choi et al. 2000). *dps* is also expressed during exponential growth, following exposure to low doses of H_2O_2, as part of the OxyR regulon, suggesting that it is critical for survival during oxidative stress (Altuvia et al. 1994).

Burkholderia pseudomallei is a facultative intracellular human pathogen that can cause a fatal disease, melioidosis. Bacterial pathogens are frequently exposed to reactive oxygen species during the course of infection. Exposure to oxygen radicals, in the form of superoxides, hydrogen peroxides, and organic hydroperoxides, can result from release of lysosomal contents within inflammatory cells or their generation by bacterial cellular metabolism (Buettner 1993; Storz and Toledano 1994). The organic hydroperoxide *tert*-butyl hydroperoxide (*t*-BOOH) has been shown to cause DNA base damage in cultured mammalian cells through its ability to react with metals to generate the highly reactive *tert*-butoxyl radical (Altman et al. 1994). Recently, an iron-binding Dps-like protein, Dpr, from *Streptococcus mutans* was found to prevent iron-dependent hydroxyl radical formation (Fenton reactions); the protein incorporates up to 480 iron and 11.2 zinc atoms per molecule (Yamamoto et al. 2002). Therefore, it is possible that the metal-binding ability of Dpr might protect cells against organic hydroperoxide toxicity by preventing hydroxyl radical formation via the Fenton reaction. Elimination of alkyl hydroperoxides is particularly important, because they are highly toxic molecules that can initiate a lipid peroxidation chain reaction that propagates free radicals, leading to DNA and membrane damage (Halliwell and Gutteridge 1984; Akaike et al. 1992). Alkyl hydroperoxide reductase (AhpR) and organic hydroperoxide re-

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sistance protein (Ohr) are the two major microbial enzymes that have been shown to be involved in the detoxification of organic hydroperoxides through the reduction of physiological lipid peroxides, such as linoleic acid hydroperoxide, thymine hydroperoxide and nonphysiological alkyl hydroperoxides, to their nontoxic alcohol forms. Genetic analysis of several bacteria has shown that mutations in the genes encoding these two enzymes lead to an organic-hydroperoxide-hypersensitive phenotype (Storz et al. 1989; Mongkolsuk et al. 1998; Fuangthong et al. 2001).

In its natural environment, whether in a human host or an external environment during transmission, it is likely that *B. pseudomallei* is faced with growth-limiting or potentially lethal conditions, such as nutrient limitation and osmotic and oxidative stress. A facultative intracellular bacterium, *B. pseudomallei* is able to grow under conditions that are usually detrimental to the development of most microorganisms. However, while the ability of *B. pseudomallei* to resist many kinds of stresses is of particular clinical importance, there is currently very little information demonstrating how *B. pseudomallei* overcomes the various stresses it encounters during infection.

In this work, we reveal the protective role of DpsA against exogenous toxic organic hydroperoxides, as well as characterize its gene regulation in response to oxidative and osmotic stresses.

Materials and methods

Bacterial strains and growth conditions

The clinical isolate *B. pseudomallei* P844, *E. coli* DH5α, and their derivatives were grown in Luria-Bertani (LB) medium. *B. pseudomallei oxyR* (R957), *katG* (G221), and *oxyR katG* (RG27) knockout strains were described in previous studies (Loprasert et al. 2002, 2003b). *E. coli* TA4315 is an AhpC-deficient strain (Storz et al. 1989). Plasmid pAhpC consists of a *B. pseudomallei ahpC* fragment in pBBR-Cm (Kovach et al. 1995) and was constructed as previously described (Loprasert et al. 2003a). Pseudomonas agar base supplemented with SR103E (cetrimide, fucidin, and cephaloridine) (Oxoid) was used after conjugation as a selective medium to inhibit growth of *E. coli*. M9 minimal medium was supplied by Gibco BRL. All cultures were grown at 37°C. Tetracycline (60 μg/ml), chloramphenicol (40 μg/ml), specinomycin (100 μg/ml), and erythromycin (100 μg/ml) were used, when required.

Northern analysis

Extraction of total RNA, using a modified hot acid phenol method, and Northern blot analysis of mRNA were carried out as previously described (Mongkolsuk et al. 1996), using a 500-bp *Sph*I-*Pst*I fragment spanning *dpsA* as the probe. Mid-exponential-phase cultures of *B. pseudomallei* were induced by the addition of menadione to a final concentration 200 μM. Both induced and uninduced cultures were then incubated for an additional 15 min prior to the isolation of total RNA.

Construction of a chromosomal *dpsA::lacZ* transcriptional fusion

A minitransposon containing the *dpsA* promoter fused to *lacZ*, pUT-*dpsA*, was constructed using the vector pUT-*Tn5lacZ1* (de Lorenzo et al. 1990). In brief, the kanamycin resistance gene was removed and replaced with the trimethoprim resistance gene (*dfr*) of pGSTp (Shalom et al. 2000). A 600-bp *Eco*RV–*Eco*RI fragment, containing the 5′ end of *dpsA* and 400 bp of upstream sequence, was blunt ended and inserted into the *Sma*I site upstream of *lacZ* in pUT-*Tn5lacZ1*. The resulting plasmid, pUT-*dpsA*, was then conjugally transferred into *B. pseudomallei* and stable trimethoprim resistant transconjugants were selected. Using this method, the *dpsA::lacZ* construct was integrated into the chromosome of both *B. pseudomallei* wild type and *oxyR* mutant strains (Loprasert et al. 2002) creating P844D and R957D.

Induction of *dpsA* promoter by salts

NaCl or KCl (500 mM final concentration) was added to overnight cultures (OD_{600}=0.5) growing in M9 medium containing low (0.05%) glucose. The induced and uninduced cultures were grown for 1 h at 37°C before being harvested for β-galactosidase assays.

β-Galactosidase assays

Cell lysates were prepared using B-PER (bacterial protein extraction reagent, Pierce) and assayed for β-galactosidase activity using *o*-nitrophenyl-β-D-galactoside (ONPG) as the substrate as previously described (Steers et al. 1965).

Construction of *dpsA* and *dpsA oxyR* mutants

The *dpsA* knockout mutant, D18, was created by insertion of a tetracycline resistant plasmid into *dpsA*. A 180-bp *Eco*RI-*Pst*I fragment from the coding region of *dpsA* was ligated into the suicide vector pKNOCK-Tc (Alexeyev 1999) to create pD180, which was then transferred from *E. coli* S17-1 λ*pir* into *B. pseudomallei* by conjugation. Mutants (D18) containing a single-crossover within *dpsA* were selected on Pseudomonas agar containing 60 μg/ml tetracycline. A *dpsA oxyR* double mutant was created by conjugation between D18 (*dpsA*) and R957 (*oxyR*), resulting the Tc^r Cm^r*dpsA oxyR* mutant strain DR17. Both D18 and DR17 were shown by Southern analysis to contain the desired gene disruptions.

Complementation of the *dpsA* and *dpsA oxyR* mutants

The *dpsA* structural gene and its ribosome-binding site (500 bp) were amplified by PCR and cloned into pGEM-T (Promega) to generate pGEM-D. The primers were D303 (5′AAGGAGTTTCGAGGATGG3′) and D304 (5′TCAC-GCGAGCAGCGAACG3′). *dpsA* was then removed from pGEM-D and cloned into the *Apa*I-*Spe*I site of pBBR-Sp (specinomycin resistant), which was created by replacing *cat* (chloramphenicol resistance gene) of pBBR-Cm with the Sp^r gene from pKRP13 (Reece and Phillips 1995), to create pDps, which was then mobilized into the *dpsA* mutant DR18 by conjugation. In order to complement the *dpsA oxyR* mutant DR17, pUT-*oxyR-ery* (erythromycin resistant) was mobilized into the chromosome of DR17 which harbors pDps, creating DR17R/pDps (*dpsA oxyR TnR*/pDps).

Growth on oxidant agar plates

Cultures of the desired strains grown overnight in M9 low glucose medium were adjusted to OD_{600}=1.0 and serially diluted. Ten microliters of each dilution was spotted onto LB agar containing 150 µM *tert*-butyl hydroperoxide (*t*-BOOH) and the extent of growth was observed after 24 h of incubation at 37°C.

Survival in oxidant medium

Overnight cultures in LB medium were subcultured (starting OD_{600}=1.0) into fresh modified M9 (0.2% casamino acids, 0.4% glucose) with and without 150 µM *t*-BOOH and the optical density was measured after 7 h of incubation at 37°C with shaking. The relative growth was calculated by comparing the optical density of treated cultures with comparable untreated cultures.

Growth inhibition zone assays

To test the susceptibility of *E. coli* strains to organic hydroperoxides, disk inhibition assays were done as previously described (Mongkolsuk et al. 1998). Briefly, bacterial cells from an exponential-phase culture (10^8 cells) were added to 3 ml of warm top LB agar. The mixture was then overlayed onto an LB agar plate. When the agar had set, 6-mm paper discs containing 6 µl of 250 µM *t*-BOOH were placed on the cell lawn. Zones of growth inhibition were measured after a 24- h incubation.

Reduction of organic hydroperoxide assay

The reduction of organic hydroperoxide in the growth medium was measured at different times by a reaction using chromogen xylenol orange, ammonium ferrous sulfate, and sulfuric acid as previously described (Shea and Mulks 2002).

Results and discussion

Regulation of *dpsA* expression by OxyR

We have previously shown that *dpsA* is co-transcribed with *katG* upon exposure to oxidative stress (Loprasert et al. 2003b) (Fig. 1A). To test whether the global peroxide sensor OxyR is a regulator of *dpsA* expression, the relative amounts of *dpsA* mRNA in *oxyR*, *katG*, and *oxyR katG* double mutant strains were determined by Northern blot analysis. A lack of OxyR in *oxyR* (R957) and *oxyR katG* (RG27) mutants abolished the induction of *dpsA* following treatment with the superoxide generator menadione (Fig. 1B lane M of *oxyR* and *oxyR katG*). Transcripts of *katG–dpsA* (3.5 kb) and *dpsA* (0.6 kb) were not induced in OxyR-deficient strains when cells were exposed to oxidant (menadione). In the *katG* mutant, a *katG–dpsA* transcript was not detected while *dpsA* mRNA was highly induced, indicating that OxyR can activate *dpsA* expression from the *dpsA* promoter (Fig. 1B lane M of *katG*). It is worthwhile noting that, in the wild-type following oxidant treatment, a transcript of approximately 3.5 kb (*katG–dpsA*) was highly induced while the 0.6-kb mRNA of *dpsA* showed no increase. *dpsA* transcripts were apparent only when *katG* was disrupted, suggesting that OxyR may preferably activate *dpsA* via the *katG* promoter instead of the downstream *dpsA* promoter. Arrangement of *katG* and *dpsA* in an operon would certainly benefit cells by allowing a prompt increase in expression of both genes in response to oxidative stress. While KatG detoxifies the peroxide threat, DpsA would simultaneously protect DNA from peroxide-induced damage.

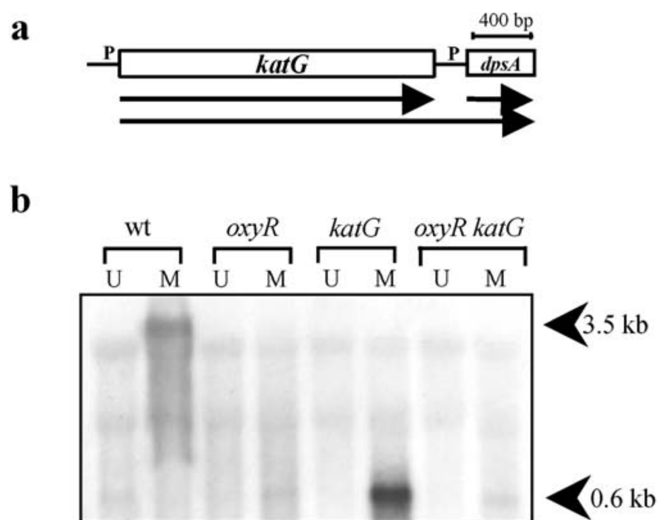

Fig. 1 Gene organization and transcriptional regulation of *katG dpsA* operon expression in response to oxidative stress. **a** The genetic organization of *katG* and *dpsA*. *Arrows* indicate the direction and extent of transcription, *P* promoter regions. **b** Northern analysis of *dpsA* mRNA prepared from *Burkholderia pseudomallei* P844 cells (wt), *oxyR* (R957), *katG* (G221), and *oxyR katG* (RG27) mutants under uninduced (U) and menadione-induced (M) conditions. *Arrowheads* indicate hybridizing mRNAs and their sizes (kb) are shown

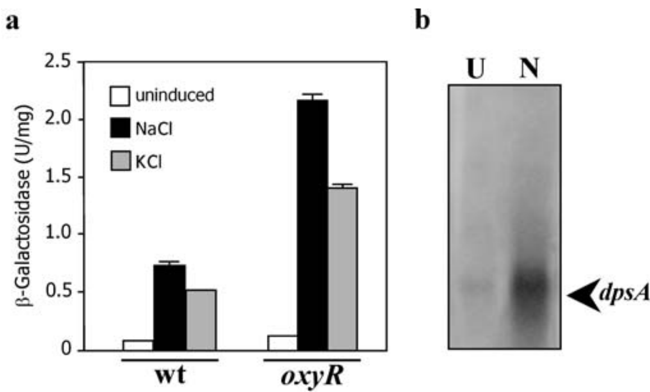

Fig. 2 Regulation of the *dpsA* promoter in response to osmotic stress. **a** β-Galactosidase activities in crude extracts of *dpsA-lacZ* fusion parent P844D (wt), and *oxyR* mutant R957D (*oxyR*) prepared from uninduced cells and cells induced with NaCl and KCl. Each value shown is the mean of three separate experiments: *error bars* standard error from the mean. **b** Northern analysis of *dpsA* mRNA prepared from *B. pseudomallei* P844 cells under uninduced (U) and NaCl-induced (N) conditions

Inducible transcription of *dpsA* by osmotic stress

To determine whether *dpsA* could be induced by oxidative and osmotic stresses, cells were treated with 0.5–10 mM H_2O_2, 500 mM NaCl or 500 mM KCl and *dpsA-lacZ* expression was monitored. Under these conditions, H_2O_2 did not cause any significant induction of the *dpsA* promoter (data not shown). Expression of *dpsA-lacZ* in wild-type (P844D) cells was induced eightfold and sixfold by NaCl and KCl, respectively, compared to an uninduced control. In the *oxyR*-disrupted mutant R957D, the level of *dpsA-lacZ* induction increased to 18-fold and 12-fold, respectively, for NaCl and KCl (Fig. 2A). Therefore, salt induction of *dpsA* expression does not require OxyR. To confirm that the *dpsA* promoter is indeed induced by salt, the relative amounts of *dpsA* mRNA in the wild- type strain under uninduced and NaCl-induced conditions were determined by Northern blot analysis. A 0.6-kb *dpsA* transcript was highly induced when cells were exposed to 500 mM NaCl for 1 h (Fig. 2B). These results are similar to those obtained in *E. coli,* where NaCl was also found to induce expression of the genes controlled by OxyR, including *dps*, in a RpoS-dependent manner (Michan et al. 1999). However, it is worth noting that *B. pseudomallei dpsA* responds more strongly to osmotic stress when OxyR is absent whereas expression in an OxyR-deficient strain of *E. coli dps* showed no effect. This leads us to speculate that in *B. pseudomallei* reduced OxyR might normally bind and repress the *dpsA* promoter in the same manner that it has previously been shown to bind to, and repress expression of, the *katG* promoter in uninduced *B. pseudomallei* (Loprasert et al. 2003b). Therefore, a lack of OxyR would certainly facilitate the RpoS dependence of the *dpsA* promoter by RNA polymerase. Expression of *dps* was also shown to be induced by general stress, e.g. heat shock, exposure to high salt or ethanol, and after glucose starvation in *B. subtilis* (Antelmann et al. 1997).

t-BOOH sensitivity of *dpsA* mutants

The physiological role of DpsA in *B. pseudomallei* was determined by testing the sensitivity of the various mutants to organic hydroperoxide stress. DpsA-deficient mutants exhibited hypersensitivity to *t*-BOOH. The *dpsA* mutant D18 did not grow well on 150 µM *t*-BOOH-containing agar while the growth of *dpsA oxyR* double mutant DR1, was even poorer. In both strains, growth was restored to the wild-type level after complementation with the *dpsA*-containing plasmid pDps (Fig. 3A). The ability of the wild-type and mutant strains to grow in M9 minimal liquid medium containing 150 µM *t*-BOOH was also studied (Fig. 3B) and the results are in good agreement with those determined on agar plates. In the wild-type strain P844, overexpression of DpsA from pDps (strain P844/pDps) increased relative growth following *t*-BOOH exposure (75% compared to 55% for wild-type). In strain D18, which lacks a functional *dpsA*, the relative growth following *t*-BOOH exposure was reduced to 36%. When either DpsA or AhpC was overexpressed in the *dpsA* mu-

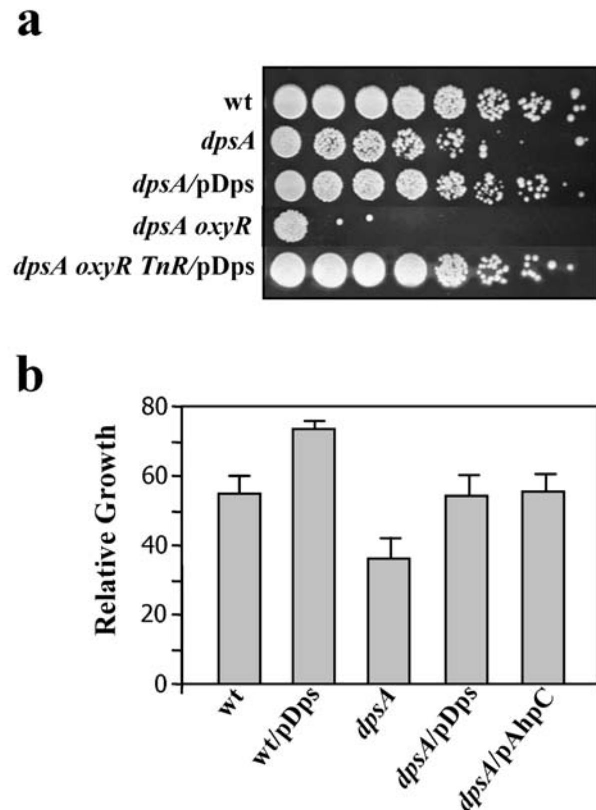

Fig. 3 Sensitivity of *B. pseudomallei* to *t*-BOOH. **a** Growth on *t*-BOOH-containing agar plate of serially diluted *B. pseudomallei* P844 (wt), *dpsA* mutant (*dpsA*), *dpsA* mutant complemented with *dpsA* on plasmid pDps (*dpsA*/pDps), *dpsA oxyR* mutant (*dpsA oxyR*), and the *dpsA oxyR* mutant complemented strain (*dpsA oxyR TnR*/pDps). **b** Relative growth of various strains in *t*-BOOH-containing M9 medium. *B. pseudomallei* P844 (wt), overexpressed DpsA (wt/pDps), *dpsA* mutant (*dpsA)*, complemented *dpsA* mutant (*dpsA*/pDps), and *dpsA* mutant with AhpC plasmid pAhpC (*dpsA*/pAhpC). Each value shown is the mean of three separate experiments; *error bars* standard error from the mean

tant strains D18/pDps and D18/pAhpC the relative rate of growth was restored to the wild-type level. This restoration of *t*-BOOH resistance in the *dpsA* strain expressing AhpC was expected since AhpC reduces and detoxifies *t*-BOOH (Storz et al. 1989). Dps-deficient *E. coli* mutants have been shown to be hypersensitive to H_2O_2 (Almiron et al. 1992), *N*-ethylmaleimide (NEM) (Ferguson et al. 1998), and acid stress (Choi et al. 2000). *B. subtilis mrgA* mutants are sensitive to H_2O_2 (Chen and Helmann 1995). To our knowledge, this is the first report demonstrating that Dps protects cells from organic hydroperoxide toxicity. We have previously found that a *B. pseudomallei katG* mutant shows increased sensitivity to H_2O_2, menadione, NEM, and sodium hypochlorite (Loprasert et al. 2003b). In order to rule out the possibility that KatG expression might be reduced in the *dpsA* mutant strain, the sensitivity of this strain to each of the aforementioned oxidants was measured. It was found that the *dpsA* mutant had the same sensitivity, as determined by growth inhibition zone assays, to H_2O_2 (0.5 M), menadione (100 mM), NEM (0.1 M), and sodium hypochlorite (0.6%) as the wild-type (data not shown).

Protection of *E. coli* against *t*-BOOH by *B. pseudomallei* DpsA

To test whether the protective property of DpsA to organic hydroperoxide is specific to *B. pseudomallei*, *dpsA* was overexpressed in the organic-oxidant-sensitive *E. coli* strain TA4315 (Storz et al. 1989) which lacks functional *ahpC*. Growth inhibition studies clearly demonstrated that both *B. pseudomallei* DpsA and AhpC could protect *E. coli* against *t*-BOOH toxicity, suggesting that this is a common property of DpsA (Fig. 4A). Since *B. pseudomallei* AhpC also conferred protection to *t*-BOOH, we were interested in finding out whether both AhpC- and DpsA-mediated protection involve the same or different mechanisms. It has been well documented that AhpC is a reductase that catalyzes the reduction of alkyl hydroperoxide to alcohol (Storz et al. 1989). The levels of *t*-BOOH in the culture medium during growth of *E. coli* strain TA4315 expressing either *B. pseudomallei* AhpC or DpsA from plasmid were therefore determined. As anticipated, the AhpC-expressing strain (TA4315/pAhpC) completely reduced the *t*-BOOH in the culture medium within 20 min, whereas the DpsA-expressing strain (TA4315/pDps) showed no significant reduction of *t*-BOOH levels relative to strain TA4315 carrying plasmid vector pBBR (Fig. 4B). This indicates that the mechanisms of protection employed by DpsA and AhpC during organic hydroperoxide exposure are distinct. It is likely that the binding of DpsA to DNA acts as a physical barrier to organic-oxidant-induced DNA damage in a manner analogous to that observed for hydrogen peroxide protection (Wolf et al. 1999). By contrast, AhpC enzymatically detoxifies the organic hydroperoxides. Analysis of *ahpC* expression in *Legionella pneumophila* and *Salmonella typhimurium* showed that *ahpC* levels increased several-fold during intracellular growth of the bacteria (Francis et al. 1997; Rankin et al. 2002), suggesting that physiological organic peroxide stress is an important threat to intracellular pathogens. Moreover, *B. pseudomallei* has also been shown to contain AhpC and KatG (catalase–peroxidase) which have the capacity to relieve a portion of the reactive nitrogen and oxidative stresses (Loprasert et al. 2003a, b).

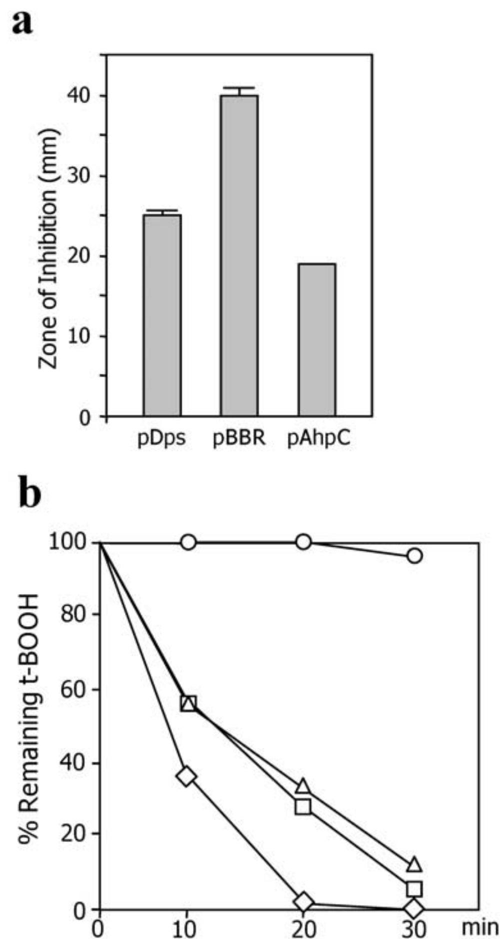

Fig. 4 Growth inhibitory zone and *t*-BOOH degradation assays. **A** Determination of the levels of resistance to *t*-BOOH killing displayed by *E. coli* TA4315 harboring pDps, pBBR vector, and pAhpC. **B** Measurement of the remaining *t*-BOOH after degradation by *Escherichia coli* TA4315 strains harboring pDps (*triangles*), pBBR vector (*squares*), and pAhpC (*diamonds*). Control (circles) is LB without cells. Each value shown in **a** and **b** is the mean of three separate experiments; *error bars* standard error from the mean.

We have uncovered a novel physiological role for DpsA in the protection against organic hydroperoxide stress. The protein acts as an additional system that can be used by *B. pseudomallei* to guard against attack by host immune systems. This study demonstrates that DpsA is a key component of the stress protection system important for the survival of the infectious pathogen *B. pseudomallei*.

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