Quorum sensing regulates *dpsA* and the oxidative stress response in *Burkholderia pseudomallei*

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Burkholderia pseudomallei is the causative agent of melioidosis, a fatal human tropical disease. The non-specific DNA-binding protein DpsA plays a key role in protecting *B. pseudomallei* from oxidative stress mediated, for example, by organic hydroperoxides. The regulation of *dpsA* expression is poorly understood but one possibility is that it is regulated in a cell population density-dependent manner via *N*-acylhomoserine lactone (AHL)-dependent quorum sensing (QS) since a *lux*-box motif has been located within the *dpsA* promoter region. Using liquid chromatography and tandem mass spectrometry, it was first established that *B. pseudomallei* strain PP844 synthesizes AHLs. These were identified as *N*-octanoylhomoserine lactone (C8-HSL), *N*-(3-oxooctanoyl)homoserine lactone (3-oxo-C8-HSL), *N*-(3-hydroxyoctanoyl)-homoserine lactone (3-hydroxy-C8-HSL), *N*-decanoylhomoserine lactone (C10-HSL), *N*-(3-hydroxydecanoyl)homoserine lactone (3-hydroxy-C10-HSL) and *N*-(3-hydroxydodecanoyl)homoserine lactone (3-hydroxy-C12-HSL). Mutation of the genes encoding the LuxI homologue BpsI or the LuxR homologue BpsR resulted in the loss of C8-HSL and 3-oxo-C8-HSL synthesis, demonstrating that BpsI was responsible for directing the synthesis of these AHLs only and that *bpsI* expression and hence C8-HSL and 3-oxo-C8-HSL production depends on BpsR. In *bpsI*, *bpsR* and *bpsIR* mutants, *dpsA* expression was substantially down-regulated. Furthermore, *dpsA* expression in *Escherichia coli* required both BpsR and C8-HSL. *bpsIR*-deficient mutants exhibited hypersensitivity to the organic hydroperoxide *tert*-butyl hydroperoxide by displaying a reduction in cell viability which was restored by provision of exogenous C8-HSL (*bpsI* mutant only), by complementation with the *bpsIR* genes or by overexpression of *dpsA*. These data indicate that in *B. pseudomallei*, QS regulates the response to oxidative stress at least in part via the BpsR/C8-HSL-dependent regulation of DpsA.

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INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis, a fatal tropical disease endemic in areas of Southeast Asia and Australia (Wuthiekanun *et al.*, 1995). The organism can be isolated from soil and water. Human infections occur mainly through skin abrasions and

inhalation of contaminated aerosols. Frequent relapse has been observed after apparent cure and serological studies have shown that a significant proportion of individuals in endemic areas can be infected asymptotically (Cheng & Currie, 2005). *In vitro* studies have demonstrated that *B. pseudomallei* can survive and multiply inside phagocytes (Jones *et al.*, 1996). To survive inside the phagolysosome, the

Abbreviations: AHL, *N*-acylhomoserine lactone; C8-HSL, *N*-octanoylhomoserine lactone; C10-HSL, *N*-decanoylhomoserine lactone; 3-hydroxy-C8-HSL, *N*-(3-hydroxydecanoyl)homoserine lactone; 3-hydroxy-C12-HSL, *N*-(3-hydroxydodecanoyl)homoserine lactone; 3-oxo-C8-HSL, *N*-(3-oxooctanoyl)homoserine lactone; LC MS/MS, liquid chromatography tandem mass spectrometry; QS, quorum sensing; *t*-BOOH, *tert*-butyl hydroperoxide.

organism has to endure both acid and oxidative stress. DNA-binding protein from starved cells (Dps) is an abundant protein in stationary-phase *Escherichia coli* cells (Almiron *et al.*, 1992). Although Dps was originally described as a non-specific DNA-binding protein involved in resistance to oxidative stress, it is actually a bacterioferritin and there are examples of Dps proteins which both bind DNA and sequester iron (Martinez & Kolter, 1997; Stillman *et al.*, 2005). These are thought to protect DNA from damage both as a physical shield and by inhibiting oxyradical formation catalysed by the Fenton reaction. Recently, the crystal structures of two Dps proteins (DpsA and DpsB) from *Lactococcus lactis* have been described; both proteins were demonstrated to bind DNA via an N-terminal α -helix (Stillman *et al.*, 2005).

In *B. pseudomallei*, DpsA has been shown to protect DNA from damage by both acid and oxidative stress (Loprasert *et al.*, 2004). The *dpsA* gene in the *B. pseudomallei* genome is located downstream of *katG*, which encodes a bifunctional enzyme with both catalase and peroxidase activities. Although the mechanism by which DpsA is regulated is not well understood, it is known that expression increases in response to oxidative stress through increased transcription of the *katG* (catalase peroxidase) promoter, which is OxyR-dependent (Loprasert *et al.*, 2004). Furthermore, *dpsA* can also be transcribed from its own promoter in an OxyR-independent manner (Loprasert *et al.*, 2004).

Quorum sensing (QS) is a term used to describe the phenomenon where bacteria coordinate the production of a diverse array of phenotypic behaviours in accordance with their cell population size via production of diffusible cell-to-cell signal molecules (Swift *et al.*, 2001; Cámara *et al.*, 2002). Once a threshold concentration has been reached, a response is triggered that leads to changes in gene expression and consequently the phenotype of the cells. In Gram-negative bacteria, the most intensively studied QS systems rely upon the interaction of *N*-acylhomoserine lactone (AHL) signal molecules, synthesized via LuxI-type AHL synthases, with LuxR-type transcriptional regulator proteins. Together, the LuxR-type protein and its cognate AHL then activate the expression of specific target genes (Swift *et al.*, 2001). Many Gram-negative bacteria possess more than one LuxR and/or LuxI gene and produce multiple AHLs. For example, the opportunistic pathogen *Pseudomonas aeruginosa* contains two LuxRI systems which operate in a hierarchical manner to regulate an arsenal of virulence determinants and secondary metabolites (Cámara *et al.*, 2002; Lazdunski *et al.*, 2004).

In *B. pseudomallei*, a LuxRI AHL-dependent QS system termed BpsRI was first described in 2002 by P. Lumjiaktase and co-workers (GenBank accession no. AF501236). Subsequently, Valade *et al.* (2004) reported that the PmlI-PmlR QS system is required for full virulence in *B. pseudomallei* strain 008 as a *pmlI* mutant was significantly less virulent than the parental strain in a murine infection model. The PmlI protein exhibits 98 % sequence identity to

BpsI (Valade *et al.*, 2004). In *B. pseudomallei* strain KHW, a LuxRI pair closely related to BpsI-BpsR was described by Song *et al.* (2005), who reported that it positively regulated phospholipase C but negatively regulated siderophore production. Both *bpsI* and *bpsR* mutants were attenuated in a *Caenorhabditis elegans* virulence assay (Song *et al.*, 2005). Using HPLC and bioassays of *B. pseudomallei* spent culture supernatants, Valade *et al.* (2004) tentatively identified *N*-decanoylhomoserine lactone (C10-HSL), which they attributed to PmlI although they did not examine the supernatant of the *pmlI* mutant or express *pmlI* in *E. coli* to establish whether PmlI was indeed responsible for C10-HSL synthesis. Song *et al.* (2005) expressed *bpsI* in *E. coli* and, by HPLC, tentatively identified *N*-octanoylhomoserine lactone (C8-HSL) but did not examine the AHL profile of a *B. pseudomallei* *bpsI* mutant. Recently, three LuxRI pairs together with two additional LuxR homologues have been identified in *B. pseudomallei* DD503 (Ulrich *et al.*, 2004a). DD503 was reported to produce at least five AHLs, including C8-HSL, C10-HSL, *N*-(3-hydroxyoctanoyl) homoserine lactone (3-hydroxy-C8-HSL), *N*-3-hydroxydecanoyl homoserine lactone (3-hydroxy-C10-HSL) and *N*-3-oxotetradecanoyl homoserine lactone (3-oxo-C14-HSL). Mutation of individual *B. pseudomallei* luxI homologues was reported to have no effect on the AHL profile (Ulrich *et al.*, 2004a).

The regulation of *dpsA* expression in *Burkholderia* is poorly understood but one possibility is that it is regulated via AHL-dependent QS since there is a *lux* box motif located within its promoter region. Here we define the nature of the AHLs synthesized by *B. pseudomallei* PP844 and show that *dpsA* expression and resistance to oxidative stress is dependent on QS via BpsIR and C8-HSL.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used are shown in Table 1. Unless otherwise stated, bacteria were cultured using Luria-Bertani (LB) broth or agar with appropriate antibiotics at 37 °C. In the case of mixed cultures, e.g. conjugations, incubations were at 30 °C. *Pseudomonas* agar base supplemented with SR 103E (cetrimide, fucidin and cephaloridine) from Oxoid was used, after conjugation, as a selective medium to inhibit growth of *E. coli*. M9 minimal medium with 2 % (w/v) glucose was used for β -galactosidase activity assays. Antibiotics were used at the following concentrations when required: ampicillin 100 $\mu\text{g ml}^{-1}$, trimethoprim 200 $\mu\text{g ml}^{-1}$ for *B. pseudomallei* and 100 $\mu\text{g ml}^{-1}$ for *E. coli*, spectinomycin 800 $\mu\text{g ml}^{-1}$ for *B. pseudomallei* and 200 $\mu\text{g ml}^{-1}$ for *E. coli*, tetracycline 60 $\mu\text{g ml}^{-1}$ and chloramphenicol 40 $\mu\text{g ml}^{-1}$.

Amplification and cloning of *bpsI* and *bpsR* genes. Using the *cepI* and *cepR* genes of *Burkholderia cepacia* as a template for the BLAST program (<http://www.ncbi.nih.gov/blast/>), homologues of this AHL synthase and its cognate transcriptional activator were identified in the *B. pseudomallei* genome database (http://www.sanger.ac.uk/Projects/B_pseudomallei/) and designated *bpsI* and *bpsR* respectively. A PCR product of 663 bp containing the full-length *bpsI* was amplified from *B. pseudomallei* strain PP844 genomic DNA using primers BPSIF (5'-CTGCAGCTCCTTGAATGACGAACGGC-3') and BPSIR (5'-AAGCTTCATGCGAACTTTCGTTTCATGG-3')

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or characteristic(s)*	Source or reference
<i>B. pseudomallei</i>		
PP844	Wild-type, virulent clinical isolate	Loprasert <i>et al.</i> (2000)
PKI5	PP844 containing pKNOCK:: <i>bpsI</i> p, Tc	This study
PKR7	PP844 containing pKNOCK:: <i>bpsR</i> p, Cm ^r	This study
KBIR5	PP844 containing pKNOCK:: <i>bpsR</i> p; pKNOCK:: <i>bpsI</i> p, Tc ^r Cm ^r	This study
PKR7 + R	PKR7 containing pBBR-R2, Cm ^r Sp ^r	This study
KBIR5 + IR	KBIR5 containing pBBR-IR3, Tc ^r Cm ^r Sp ^r	This study
KBIR5 + <i>dpsA</i>	KBIR5 containing pDps, Tc ^r Cm ^r Sp ^r	This study
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 hsdR17</i> ($r_k^- m_k^+$) <i>supE44</i> λ^- <i>thi-1</i> <i>relA1</i> <i>gyrA96</i>	Hanahan (1983)
S17-1 λ pir	RP4-2- <i>tet</i> ::Mu-1 <i>kan</i> ::Tn7 (Tp ^r Sm ^r) <i>thi proA hsdR recA</i>	Simon <i>et al.</i> (1983)
CC118	Strain used as a host for conjugation of transposable element pUT-mini-Tn5Cm	De Lorenzo <i>et al.</i> (1990)
CpUT	CC118 containing TnpD vector, Tp ^r	Loprasert <i>et al.</i> (2004)
CpUT + R	CpUT containing pBBR-R2, Tp ^r Sp ^r	This study
EBPI	DH5 α containing pUCI, Amp ^r	This study
Plasmids		
pUCI	pUC19 containing the full-length <i>B. pseudomallei bpsI</i> gene, Amp ^r	This study
pKNOCK-Tc	Mobilizable suicide vector for construction of gene knockouts in Gram-negative bacteria, Tc ^r	Alexeyev (1999)
pKNOCK-Cm	Mobilizable suicide vector for construction of gene knockouts in Gram-negative bacteria, Cm ^r	Alexeyev (1999)
pBBR-Sp	Broad-host-range cloning vector, Sp ^r	Loprasert <i>et al.</i> (2004)
pKBI	pKNOCK containing a 298 bp internal segment of <i>B. pseudomallei bpsI</i>	This study
pKBR	pKNOCK containing a 323 bp internal segment of <i>B. pseudomallei bpsR</i>	This study
pBBR-R2	pBBR-Sp containing full-length <i>B. pseudomallei bpsR</i>	This study
pBBR-IR3	pBBR-Sp containing full-length <i>B. pseudomallei bpsIR</i>	This study
pDps	pBBR-Sp containing full-length <i>B. pseudomallei dpsA</i>	Loprasert <i>et al.</i> (2004)
TnpD	A 654 bp fragment containing the 5' end of <i>dpsA</i> and 400 bp of upstream <i>dpsA</i> sequence inserted upstream of <i>lacZ</i> in pUT-Tn5 <i>lacZ</i> 1, Tp ^r	Loprasert <i>et al.</i> (2004)

*Amp^r, ampicillin resistant; Tc^r, tetracycline resistant; Cm^r, chloramphenicol resistant; Sp^r, spectinomycin resistant; Tp^r, trimethoprim resistant.

and cloned into the *Hind*III and *Pst*I sites of pUC19 to create pUCI. A PCR product of 2.5 kbp containing both *bpsI* and *bpsR* (*bpsIR*) was amplified using primers BPSIF and BPSRR (5'-CTGCAGG-AACCGTTGATGGAGTGAGC-3') and cloned into pBBR-Sp by blunt-ended ligation to create pBBR-IR3. The 2.5 kbp PCR product was cut by *Eco*RI to obtain a 1288 bp DNA fragment containing full-length *bpsR* gene; this was cloned into pBBR-Sp to create pBBR-R2. The sequences of *bpsI* and *bpsR* from *B. pseudomallei* strain PP844 were deposited in GenBank (accession no. AF501236).

Construction of *B. pseudomallei bpsI* and *bpsR* knockout and complemented mutants. *bpsI* (PKI5), *bpsR* (PKR7) and *bpsIR* (KBIR5) mutants were constructed in *B. pseudomallei* strain PP844. Briefly, A 298 bp fragment of the *bpsI* gene was amplified from PP844 using primers BIPF (5'-GTCACGCCGATCAGTT-GCTT-3') and BIPR (5'-AGTACGATCGCGACGATACC-3'). The blunt-ended product was ligated into the suicide vector pKNOCK-Tc to create pKBI, which was then mobilized from *E. coli* S17-1 λ pir into PP844 by conjugation. Single-crossover insertion mutants were selected on pseudomonas base agar containing 60 μ g tetracycline ml⁻¹. A blunt-ended 323 bp fragment of the *bpsR* gene was amplified using primers BRPF (5'-CGACACCTATCCGAACGGCT-3') and BRPR (5'-AACGGCTCATCAGCGAGTGC-3'). The resulting fragment was ligated into pKNOCK-Cm to create pKBR. After

conjugation into PP844, mutants were selected on pseudomonas base agar containing 40 μ g chloramphenicol ml⁻¹. Finally, the double *bpsIR* mutant KBIR5 was created by conjugating pKBR into the PKI5 mutant and selecting on pseudomonas base agar containing 60 μ g tetracycline ml⁻¹ and 40 μ g chloramphenicol ml⁻¹. For complementation of the knockout strain, pBBR-R2 was conjugated into PKR7 to create PKR7 + R, which was selected on pseudomonas base agar containing 800 μ g spectinomycin ml⁻¹ and 40 μ g chloramphenicol ml⁻¹. Plasmid pBBR-IR3 was conjugated into KBIR5 to create KBIR5 + IR, which was selected on pseudomonas base agar containing 800 μ g spectinomycin ml⁻¹, 60 μ g tetracycline ml⁻¹ and 40 μ g chloramphenicol ml⁻¹.

Construction of *dpsA::lacZ* transcriptional fusion strains.

TnpD is a mini-transposon vector containing the *dpsA* promoter fused to *lacZ* and maintained in *E. coli* CC118 (CpUT) as described in previous studies (Loprasert *et al.*, 2004). Integration of the *dpsA* promoter::*lacZ* transcriptional fusion into the chromosome of *B. pseudomallei* PP844, the QS mutants PKI5, PKR7 and KBIR5 and their corresponding complemented strains was achieved by conjugation of TnpD on plates containing trimethoprim (200 μ g ml⁻¹). In order to express the DpsA protein in KBIR5, pDps (Loprasert *et al.*, 2004) was transformed into this mutant to create KBIR5 + *dpsA*. To determine whether BpsR regulated *dpsA* directly, pBBR-R2 was

introduced into *E. coli* CpUT to generate *E. coli* CpUT + R. The bacteria were selected on agar containing trimethoprim (100 µg ml⁻¹) and spectinomycin (200 µg ml⁻¹).

Assay for β -galactosidase activity. Cell lysates taken from different phases of growth of *B. pseudomallei* strains grown in MM9 medium with 0.5 µM NaCl and with or without C8-HSL (200 nM) at 37 °C were prepared using bacterial protein extraction reagent (Pierce) and assayed for β -galactosidase activity in Miller units using *o*-nitrophenyl- β -D-galactoside as a substrate (Miller, 1972). Similar assays were undertaken for *E. coli* CpUT and CpUT + R grown in the absence or presence of C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL, C10-HSL, 3-hydroxy-C10-HSL and 3-hydroxy-C12-HSL (100 nM).

Growth on oxidant agar plates. Bacterial cultures were grown overnight in M9 low-glucose medium, adjusted to OD₆₀₀ 1.0 and 10-fold serially diluted. Ten microlitres 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 incubation at 37 °C (Loprasert *et al.*, 2004).

Growth inhibition zone assay. Bacterial cultures grown overnight in M9 low-glucose medium were adjusted to OD₆₀₀ 1.0 and added to 3 ml warm top LB agar. The mixtures were overlaid onto LB agar plates. Paper discs containing *t*-BOOH (250 µM) were placed on the cell lawn. The diameters of growth inhibition zones were measured after 24 h incubation (Loprasert *et al.*, 2004).

Synthesis of AHLs. A range of AHLs with acyl side chains from C₄ to C₁₄ in length, with or without 3-oxo or 3-hydroxy substituents, were synthesized as described by Chhabra *et al.* (1993, 2003).

AHL extraction and LC MS/MS analysis. *B. pseudomallei* strains were grown to OD₆₀₀ 1.6 in 2 l tryptic soy broth at 37 °C with shaking at 250 r.p.m. Cells were removed by centrifugation and the supernatant was extracted twice with equal volumes of acidified ethyl acetate (100 µl glacial acetic acid per litre of ethyl acetate) and concentrated by rotary evaporation at 40–45 °C. The residue was resuspended in 50 µl methanol prior to liquid chromatography tandem mass spectrometry (LC-MS/MS). AHLs were separated by reverse-phase chromatography (RP-HPLC) using an Exsil Pure C18 MS 5µ column (250 × 2.1 mm; Alltech Associates) coupled to a tandem mass spectrometer (Applied Biosystems 4000 Q-TRAP) and eluted with a 35–70 % w/v acetonitrile/water gradient as described by Yates *et al.* (2002). Enhanced product trap experiments (EPI) were triggered by precursor ion (*m/z* 102) scanning for the *m/z* range 150–350. The precursor ion *m/z* 102 is characteristic of the

homoserine lactone ring moiety. EPI spectra (*m/z* range 80–400) containing an ion at *m/z* 102 were compared with the product mass spectra of the corresponding synthetic AHL standard.

RESULTS

B. pseudomallei strain PP844 produces multiple AHLs

Spent stationary-phase culture supernatants prepared from *B. pseudomallei* strain PP844 were extracted with ethyl acetate and subjected to LC MS/MS. The data obtained are summarized in Table 2. Six AHLs were unequivocally identified by comparison of their retention times, and their molecular and principal fragment ions with synthetic standards. These AHLs were C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL, C10-HSL, 3-hydroxy-C10-HSL and 3-hydroxy-C12-HSL.

C8-HSL and 3-oxo-C8-HSL production is dependent on *bpsI* and *bpsR*

The gene encoding the LuxI homologue *bpsI* was cloned from the *B. pseudomallei* PP844 chromosome and sequenced. *bpsI* is 97 % and 98 % identical to the corresponding genes from *B. pseudomallei* strains K96243 and KHW respectively (data not shown). Since the identity of the AHL(s) synthesized via BpsI have not been unequivocally chemically identified, the *bpsI* gene was expressed in *E. coli* EBPI. Ethyl acetate extracts of EBPI culture supernatants were subjected to LC MS/MS and a molecule with *m/z* 228 was identified with fragmentation ions of *m/z* 127 and 102, characteristic of C8-HSL (Table 2).

To evaluate the impact of *bpsI* and *bpsR* mutations on the AHL profile of *B. pseudomallei* PP844, we constructed *bpsI* (PKI5) and *bpsR* (PKR7) mutants. Table 2 compares the AHL profiles derived from LC MS/MS analysis of the corresponding spent culture supernatants. In both mutants, the only compounds absent are C8-HSL and 3-oxo-C8-HSL, a finding which indicates that *bpsI* is responsible for their synthesis in *B. pseudomallei* PP844 and that the BpsIR system does not affect the expression of the other AHL

Table 2. AHL profiles, by LC MS/MS analysis, of the spent culture supernatants of *B. pseudomallei* PP844, its QS mutants, and an *E. coli* strain harbouring *bpsI*

AHL	Structure	<i>m/z</i>	Retention time (min)	Principal fragment ions	PP844 (wild-type)	PKI5 (BpsI ⁻)	PKR7 (BpsR ⁻)	EBPI (<i>E. coli</i> + <i>bpsI</i>)
C8-HSL		228	5.9	228, 127, 102	+	–	–	+
3-Oxo-C8-HSL		242	4.8	242, 141, 102	+	–	–	–
3-Hydroxy-C8-HSL		244	4.6	244, 226, 125, 102	+	+	+	–
C10-HSL		256	8.5	256, 155, 102	+	+	+	–
3-Hydroxy-C10-HSL		272	5.4	272, 254, 153, 102	+	+	+	–
3-Hydroxy-C12-HSL		300	7.9	300, 282, 181, 102	+	+	+	–

synthases present in this organism. Table 2 also shows that *bpsI* is regulated by BpsR, since mutation of *bpsR* results in the loss of C8-HSL and 3-oxo-C8-HSL synthesis.

Expression of *dpsA* in *B. pseudomallei* is BpsIR/C8-HSL-dependent

In the promoter region (−74 to −55) of *dpsA*, we identified a 20 bp sequence (GCATCCCGcATCGGcATGC) as a *lux* box motif characteristic of genes which are regulated via LuxRI/AHL-dependent QS. Without the lower-case c, this motif will be perfectly palindromic. Nevertheless, this motif matches the consensus sequence for the *Vibrio fischeri luxI lux* box at 11 out of 21 positions as well as the *P. aeruginosa rhII lux* box (12/20 bases). To assess whether QS is involved in regulating the response of *B. pseudomallei* to oxidative stress, we first introduced a *dpsA::lacZ* transcriptional fusion via TnpD onto the chromosome of *B. pseudomallei* PP844, the isogenic *bpsI* (PKI5), *bpsR* (PKR7) mutants and the *bpsIR* double mutant (KBIR5) as well as the corresponding complemented strains.

Fig. 1 shows that *dpsA* expression is induced in the late exponential phase of growth (6 h post-inoculation). The *bpsI*, *bpsR* and *bpsIR* mutants all exhibited substantially reduced levels of β -galactosidase activity throughout growth when compared to the PP844 wild-type, indicating that *dpsA* is regulated via *bpsIR*. Provision of exogenous, synthetic C8-HSL to the *bpsI* mutant (PKI5) or genetic complementation of the *bpsR* (PKR7) and *bpsIR* (KBIR5) mutants completely restored *dpsA* expression (Fig. 1), suggesting that *dpsA* is directly or indirectly regulated by the *bpsIR* QS system. Fig. 1 also demonstrates that mutations in the *bpsIR* QS system have no adverse effects on the growth of *B. pseudomallei* under these culture conditions and that exogenous synthetic C8-HSL is unable to overcome the growth-phase dependency of *dpsA* expression.

B. pseudomallei bpsIR QS mutants show increased sensitivity to oxidative stress due to reduction of *dpsA* expression

Mutation or overexpression of *dpsA* in *B. pseudomallei* confers hypersensitivity or increased resistance respectively to organic oxidants such as the organic hydroperoxide *t*-BOOH (Loprasert *et al.*, 2004). To determine the sensitivity of the *bpsI*, *bpsR* and *bpsIR* mutants to oxidative stress, each strain was grown on oxidant agar plates containing 150 μ M *t*-BOOH. Each of the mutants was more sensitive to *t*-BOOH than the wild-type or the corresponding complemented strains (Fig. 2a). The wild-type and complemented strains grew when diluted to 10^{-6} – 10^{-7} c.f.u. ml $^{-1}$; however, in contrast, the QS mutants only grew when diluted to 10^{-3} – 10^{-4} c.f.u. ml $^{-1}$. This suggests that PKI5, PKR7 and KBIR5 are 1000–10 000 times more sensitive to hydroperoxide stress.

To evaluate whether the increased sensitivity of the QS mutants was a consequence of reduced *dpsA* expression, we analysed the response of the wild-type PP844, *bpsIR* mutant KBIR5 and KBIR5 carrying pDps (KBIR5 + *dpsA*). Plasmid pDps carries a copy of *dpsA* and was previously shown to enhance the resistance of *B. pseudomallei* to *t*-BOOH (Loprasert *et al.*, 2004). Fig. 2(b) shows that the wild-type grew to the dilution of 10^{-7} c.f.u. ml $^{-1}$ and KBIR5 to 10^{-2} c.f.u. ml $^{-1}$, whereas KBIR5 + *dpsA* grew to 10^{-6} c.f.u. ml $^{-1}$. Taken together, these results demonstrate that the *bpsI* and *bpsR* QS mutants are more sensitive to oxidative stress and this is likely to be due to a reduction in *dpsA* expression and hence DpsA production. The growth inhibition zone assay (Fig. 2c) further confirmed that both wild-type and complemented *B. pseudomallei* strains were more resistant to *t*-BOOH than were the QS mutants on LB agar. The *dpsA*-complemented strain also showed more resistance to *t*-BOOH, as expected.

Fig. 1. Expression of *dpsA* promoter during growth of *B. pseudomallei*. β -Galactosidase activities (means $\pm$ range) from triplicate experiments in crude extracts of the *dpsA-lacZ* transcription fusion integrated into *B. pseudomallei* parent strain PP844, *bpsI* knockout mutant (PKI5), *bpsI* knockout mutant supplied with 200 nM exogenous C8-HSL (PKI5 + C8), *bpsR* mutant (PKR7), *bpsR* knockout mutant complemented with *bpsR* plasmid pBBR-R2 (PKR7 + R), *bpsIR* double knockout mutant (KBIR5) and *bpsIR* double knockout mutant complemented with the *bpsIR* plasmid pBBR-IR3 (KBIR5 + IR) are shown by the patterned bars. The negative control used was the PP844 parent strain without the *dpsA-lacZ* transcriptional fusion. Growth (OD_{600}) is shown by the symbols and graph lines.

Fig. 2. Sensitivity of *B. pseudomallei* QS mutants to *t*-BOOH: determination of the levels of resistance to *B. pseudomallei* *t*-BOOH killing in the parent strain PP844, PKI5, PKI5 with exogenous C8-HSL (PKI5+C8), PKR7, PKR7+R, KBIR5, KBIR5+IR and KBIR5+dpsA. (a, b) Growth on oxidant agar plates assay. Serial 10-fold dilutions of cultures were spotted. (c) Growth inhibition zone assay. The diameters of the growth inhibition zone are shown (means $\pm$ range of triplicate assays).

BpsR and C8-HSL are required for maximum expression of *dpsA* in *E. coli*

To determine whether BpsR directly regulated the expression of *dpsA* in the presence or absence of AHLs, we used *E. coli* CpUT harbouring the *dpsA::lacZ* transcriptional fusion plasmid, TnpD, together with pBBR-R2 to give *E. coli* strain

CpUT+R. Fig. 3 shows that *dpsA* promoter activity in *E. coli* CpUT is ~ 800 Miller units ml^{-1} and remains unchanged on introducing *bpsR* (*E. coli* CpUT+R). Exogenous provision of C8-HSL to *E. coli* CpUT+R but not *E. coli* CpUT increased *dpsA* expression approximately threefold (to ~ 2200 Miller units ml^{-1}). None of the other AHLs produced by *B. pseudomallei* strain PP844 enhanced *dpsA* expression.

DISCUSSION

In common with Gram-negative bacteria such as *P. aeruginosa* (Winson *et al.*, 1995), *Rhizobium leguminosarum* (Lithgow *et al.*, 2000) and *Yersinia pseudotuberculosis* (Atkinson *et al.*, 1999), *B. pseudomallei* possesses several LuxI homologues and produces multiple AHL QS signal molecules. *B. pseudomallei* PP844 is an extremely virulent strain isolated from a patient who died from the most severe clinical manifestation of melioidosis (Utaisincharoen *et al.*, 2001). PP844 produces six AHLs with C₈, C₁₀ and C₁₂ acyl side chains with or without C-3 position substituents. Of these, 3-oxo-C8-HSL and 3-hydroxy-C12-HSL have not previously been identified in *B. pseudomallei* while C8-HSL, 3-hydroxy-C8-HSL, C10-HSL and 3-hydroxy-C10-HSL were previously reported by Ulrich *et al.* (2004a) in *B. pseudomallei* strain DD03. This strain also made 3-oxo-C14-HSL, an AHL which was not present in *B. pseudomallei* PP844 culture supernatants. In bacteria which possess multiple LuxRI homologues, these QS systems are usually interdependent. In *B. pseudomallei* DD03, mutation of any of the three individual AHL synthase genes had no effect on the AHL profile apart from the *pmlI* mutant, which did not produce 3-hydroxy-C14-HSL. These data do not however define which AHLs are synthesized by which LuxI homologue and suggest that there is substantial redundancy in the system. Here we have shown that mutation of *bpsI* results in the specific loss of two AHLs, C8-HSL and 3-oxo-C8-HSL, from the AHL profile of the parental PP844 strain. To confirm these data, *bpsI* was expressed in *E. coli*. However, only C8-HSL was synthesized, suggesting either that *E. coli* is unable to synthesize 3-oxo-C8-HSL via BpsI or that 3-oxo-C8-HSL is produced via a different AHL synthase, the expression of which depends on the presence of C8-HSL. When expressed in a heterologous host, LuxI homologues do not always generate the same AHL profile as in the original bacterium (Atkinson *et al.*, 1999) and this is the most likely explanation for our observation. Our unequivocal demonstration that BpsI directs the synthesis of C8-HSL is consistent with the HPLC and bioassay data reported by Song *et al.* (2005) for *B. pseudomallei* strain KHW. However, it is not possible to conclude that C10-HSL is the main AHL produced via PmlI (the equivalent gene to *bpsI*) in *B. pseudomallei* strain 088 since the authors only examined culture supernatants from the parent strain (Valade *et al.*, 2004), which produces multiple AHLs.

In *B. pseudomallei* and the closely related obligate animal pathogen *Burkholderia mallei*, QS mutants are highly

Fig. 3. Expression of *dpsA* in *E. coli* in the presence and absence of *bpsR* and AHLs (100 nM). The β -galactosidase activities were determined for *E. coli* carrying the *dpsA* :: *lacZ* fusion without (*E. coli* CpUT) or with *bpsR* (*E. coli* CpUT + R) in the absence or presence of the AHLs produced by *B. pseudomallei* PP844. C8, C8-HSL; C8=O, 3-oxo-C8-HSL; C10, C10-HSL; C12-OH, 3-hydroxy-C12-HSL; C8-OH, 3-hydroxy-C8-HSL; C10-OH, 3-hydroxy-C10-HSL. Data are means $\pm$ range of triplicate experiments.

attenuated in experimental animal infection models (Ulrich *et al.*, 2004a, b). Although *B. mallei* appears to possess only two *luxI* homologues, nevertheless it also produces C8-HSL, 3-hydroxy-C8-HSL, C10-HSL and 3-hydroxy-C10-HSL (Ulrich *et al.*, 2004b) whereas the non-pathogenic *Burkholderia thailandensis* does not produce any of the 3-hydroxy or 3-oxo compounds although it does synthesize C6-HSL, C8-HSL and C10-HSL (Ulrich *et al.*, 2004c). It is therefore possible that the QS systems employing the substituted AHLs are more closely associated with the regulation of virulence.

Mutation of *bpsR* in PP844 also resulted in the loss of C8-HSL and 3-oxo-C8-HSL synthesis, indicating that BpsR is required for the synthesis of these two AHLs, presumably by controlling *bpsI* expression. Indeed, Song *et al.* (2005) have shown that C8-HSL is required to activate transcription of both *bpsI* and *bpsR*. Our data also indicate that the *bpsIR* system does not control the expression of the two other *luxI* homologue systems present in *B. pseudomallei* although it remains possible that the other LuxR proteins and AHLs may influence *bpsIR* expression.

The organic hydroperoxide *t*-BOOH has been shown to cause DNA damage in mammalian cells because it reacts with metals to generate *tert*-butoxyl radicals (Altman *et al.*, 1994). DpsA-type proteins have previously been demonstrated to prevent iron-dependent hydroxy radical formation (Yamamoto *et al.*, 2002) and in *B. pseudomallei*, DpsA conferred protection against *t*-BOOH (Loprasert *et al.*, 2004). As *bpsI* and *bpsR* mutants exhibit reduced expression

of *dpsA*, we thought it likely that they would show increased sensitivity to *t*-BOOH. This was indeed the case, with both mutants being more sensitive to *t*-BOOH. This defect could be complemented by provision of C8-HSL to the *bpsI* mutant or by genetic complementation of the *bpsR* and *bpsRI* mutants. In addition, the viability of the QS mutants was reduced in the presence of *t*-BOOH when compared with the parent strain cultured under similar conditions. Protection against *t*-BOOH could also be achieved in the *bpsRI* mutants by increasing the expression of *dpsA*. The data suggest that the increased sensitivity to *t*-BOOH observed in the *bpsRI* mutants is due specifically to a reduction in *dpsA* expression. Thus the response of the *B. pseudomallei* wild-type to oxidative stress is partially controlled in a cell population density dependent manner through QS as demonstrated in this study, perhaps reflecting the need to protect DNA from oxidative damage in high-density 'overcrowded' stationary-phase cultures. In *P. aeruginosa*, the response to oxidative stress imposed by hydrogen peroxide and the O₂⁻-generating agent phenazine methosulphate is also QS controlled since *sodA*, *sodB* and *kata* are regulated by both the *las* and *rhl* QS systems (Hassett *et al.*, 1999).

B. pseudomallei can resist phagocytic intracellular killing (Egan & Gordon, 1996) and remain dormant within a host for many years (Nathan *et al.*, 2005). It has evolved a variety of mechanisms to protect its DNA from oxidative damage from either cellular metabolism or the environment, and under such conditions will produce high levels of the non-specific DNA-binding protein DpsA, which effectively

protects DNA against oxidants (Almiron *et al.*, 1992; Loprasert *et al.*, 2004). In *B. pseudomallei* (and also *Burkholderia cenocepacia* strain J2315) *dpsA* is located adjacent to *katG*. In the former, the two genes are co-transcribed during oxidative stress but under conditions where *katG* is not highly induced, *dpsA* is transcribed from a second promoter within the *katG*–*dpsA* intergenic region (Loprasert *et al.*, 2003). This region also contains a *lux* box motif and here we have shown that *dpsA* expression is positively controlled by the BpsRI QS system. In *B. cepacia* ATCC 25416, Aguilar *et al.* (2003) identified a genomic clone (P80) that was activated in an *E. coli* strain carrying CepR when supplied with C8-HSL. Although they were unable to identify the target gene(s) regulated by CepR in ATCC 25416, from the sequence data obtained they noted that a DpsA homologue was present 200 bp downstream of the identified sequence in *B. cenocepacia* J2315, a strain whose genome has been sequenced. Although no direct evidence was presented, it is possible that the response of *B. cepacia* complex to oxidative stress may also be QS controlled.

In *B. pseudomallei*, *dpsA* expression is not completely dependent on *bpsRI* since β -galactosidase activities of ~ 450 Miller units ml^{-1} are observed in the QS mutants (Fig. 1). It is therefore likely that *dpsA* expression is also subject to control by a number of regulatory systems where QS provides the population density signal required to trigger *dpsA* expression in combination with other environmental signals. This is a characteristic of many AHL-dependent QS systems (Withers *et al.*, 2001). Furthermore, it is noteworthy that *dpsA* expression was not advanced in *B. pseudomallei* by provision of exogenous C8-HSL and remained population and growth-phase dependent. This phenomenon has also been noted in *P. aeruginosa*, where provision of exogenous AHLs at the start of growth does not induce early induction of QS-dependent virulence determinants (Diggle *et al.*, 2002, 2003).

In conclusion, we show that (a) *B. pseudomallei* PP844 synthesizes six AHLs, two of which (3-oxo-C8-HSL and 3-hydroxy-C12-HSL) have not previously been identified in *B. pseudomallei*; (b) BpsI directs the synthesis of C8-HSL and 3-oxo-C8-HSL; and (c) BpsR, in conjunction with C8-HSL, contributes to the oxidative stress response by positively regulating *dpsA* expression.

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