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N-Octanoylhomoserine lactone signalling mediated by the BpsI–BpsR quorum sensing system plays a major role in biofilm formation of *Burkholderia pseudomallei*

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The genome of *Burkholderia pseudomallei* encodes three acylhomoserine lactone (AHL) quorum sensing systems, each comprising an AHL synthase and a signal receptor/regulator. The BpsI–BpsR system produces *N*-octanoylhomoserine lactone (C8HL) and is positively auto-regulated by its AHL product. The products of the remaining two systems have not been identified. In this study, tandem MS was used to identify and quantify the AHL species produced by three clinical *B. pseudomallei* isolates – KHW, K96243 and H11 – three isogenic KHW mutants that each contain a null mutation in an AHL synthase gene, and recombinant *Escherichia coli* heterologously expressing each of the three *B. pseudomallei* AHL synthase genes. BpsI synthesized predominantly C8HL, which accounted for more than 95 % of the extracellular AHLs produced in stationary-phase KHW cultures. The major products of $BpsI_2$ and $BpsI_3$ were *N*-(3-hydroxy-octanoyl)homoserine lactone (OHC8HL) and *N*-(3-hydroxy-decanoyl)homoserine lactone, respectively, and their corresponding transcriptional regulators, $BpsR_2$ and $BpsR_3$, were capable of driving reporter gene expression in the presence of these cognate lactones. Formation of biofilm by *B. pseudomallei* KHW was severely impaired in mutants lacking either BpsI or BpsR but could be restored to near wild-type levels by exogenous C8HL. $BpsI_2$ was not required, and $BpsI_3$ was partially required for biofilm formation. Unlike the *bpsI* mutant, biofilm formation in the *bpsI*$_3$ mutant could not be restored to wild-type levels in the presence of OHC8HL, the product of $BpsI_3$. C8HL and OHC8HL had opposite effects on biofilm formation; exogenous C8HL enhanced biofilm formation in both the *bpsI*$_3$ mutant and wild-type KHW while exogenous OHC8HL suppressed the formation of biofilm in the same strains. We propose that exogenous OHC8HL antagonizes biofilm formation in *B. pseudomallei*, possibly by competing with endogenous C8HL for binding to BpsR.

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INTRODUCTION

Burkholderia pseudomallei, a Gram-negative soil bacillus, is the causative agent of melioidosis, a severe and potentially fatal disease endemic to South-East Asia and northern Australia. The clinical presentations of melioidosis are varied, with pneumonia, abscess formation, septicaemia, genito-urinary infection and encephalomyelitis being commonly encountered (Cheng & Currie, 2005; Wiersinga *et al.*, 2006). The bacterium is classified as a Category B organism by the US Center for Disease Control and Prevention (http://www.cdc.gov). Its intrinsic resistance to many antibiotics, the recurrence of infection despite prolonged

Abbreviations: AHL, acylhomoserine lactone; C6HL, *N*-hexanoylhomoserine lactone; C8HL, *N*-octanoylhomoserine lactone; C10HL, *N*-decanoylhomoserine lactone; d_3-C6HL, *N*-hexanoyl-L-homoserine lactone-d_3; OHC8HL, *N*-(3-hydroxy-octanoyl)homoserine lactone; OHC10HL, *N*-(3-hydroxy-decanoyl)homoserine lactone; OHC12HL, *N*-(3-hydroxy-dodecanoyl)homoserine lactone; oxoC8HL, *N*-(3-oxo-octanoyl)homoserine lactone; qRT-PCR, quantitative real-time PCR; QS, quorum sensing; SRM, select reaction monitoring.

Two supplementary tables detailing the nucleotide sequences of primers used for PCR amplification and for quantitative real-time PCR are available with the online version of this paper.

antibiotic therapy and relatively high rate of mortality despite treatment are some concerns in the treatment of melioidosis (White, 2003).

Quorum sensing (QS) is a mechanism for intercellular communication commonly employed by bacteria, involving small diffusible molecules as pheromones. It allows the regulation of gene expression across the entire bacterial community in response to cell-density-dependent cues. The pheromones involved in QS in Gram-negative bacteria are usually acylhomoserine lactones (AHLs), which are amphipathic molecules derived from fatty acids (Fuqua & Greenberg, 2002). The archetype of AHL QS is the LuxI–LuxR circuitry present in the marine bacterium *Vibrio fischeri*, where this signalling system triggers the onset of luminescence at high cell densities (Fuqua *et al.*, 1994; Nealson *et al.*, 1970). The *luxI* gene encodes an AHL synthase that is weakly transcribed at low cell density, producing a basal amount of AHLs. These diffuse out of the cells and gradually accumulate in the growth medium in a cell-density-dependent manner. At a sufficiently high cell density AHLs bind to the receptor protein LuxR, and this complex acts as a transcriptional activator of target genes, including *luxI*; the ensuing positive auto-induction loop enforces a community-wide synchronous change in gene expression upon reaching the quorum (Fuqua *et al.*, 1994).

The BpsI–BpsR QS system in *B. pseudomallei* employs a similar mechanism to regulate target gene expression (Song *et al.*, 2005; Valade *et al.*, 2004). BpsI, an AHL synthase, produces *N*-octanoylhomoserine lactone (C8HL), which, upon binding the LuxR homologue BpsR, converts the latter into a transcriptional activator driving the expression of *bpsI*, *bpsR* and other target genes. This QS system controls expression of the oxidative stress protein DpsA, and the secretion of one or more uncharacterized metalloproteases, siderophores and phospholipases (Lumjiaktase *et al.*, 2006; Song *et al.*, 2005; Valade *et al.*, 2004). The genome of *B. pseudomallei* contains two other *luxI–luxR* homologue pairs, and two orphan *luxR* homologues not associated with any AHL synthases. We adopted the same nomenclature for these homologues as Kiratisin & Sanmee (2008), who also provided a detailed description of the genetic organization of each QS system, including the location of putative *lux* boxes (Kiratisin & Sanmee, 2008). The same study also attempted to dissect the transcriptional interplay between the different QS systems mediated by the LuxR homologues in the presence of a limited repertoire of AHLs. Little is known about the AHL products of the remaining two QS systems and the phenotypes they control. There is also poor consensus on the AHL species produced by *B. pseudomallei*. Up to seven different species have been reported, including C8HL, *N*-(3-hydroxy-octanoyl)homoserine lactone (OHC8HL), *N*-(3-hydroxy-decanoyl)homoserine lactone (OHC10HL), *N*-decanoylhomoserine lactone (C10HL), *N*-(3-oxo-octanoyl)homoserine lactone (oxoC8HL), *N*-(3-hydroxy-dodecanoyl)homoserine lactone (OHC12HL) and *N*-(3-oxo-tetradecanoyl)homoserine lactone, and their relative abundance has not been determined (Lazar Adler *et al.*, 2009).

Biofilms are highly structured, matrix-enclosed communities (Stoodley *et al.*, 2002), and have been shown to display an increased tolerance to antimicrobials and heavy metals (Ceri *et al.*, 1999; Teitzel & Parsek, 2003). *B. pseudomallei* is capable of forming significant amounts of biofilm when grown in liquid culture (Taweechaisupapong *et al.*, 2005; Vorachit *et al.*, 1995). Direct examination of infected tissue from humans and animals has also revealed the presence of aggregated cell clusters enclosed within a glycocalyx *in vivo* (Vorachit *et al.*, 1995). Although the extent of biofilm formation does not seem to correlate with virulence in a mouse model of infection, *B. pseudomallei* biofilms have a much increased resistance to first-line antibiotics used in the treatment of melioidosis (Vorachit *et al.*, 1993, 1995). The ability to form biofilms is therefore hypothesized to contribute to the persistence of *B. pseudomallei* infections, and melioidosis is considered a biofilm infection (Costerton *et al.*, 1999).

In this study, we re-analysed the AHL molecules present in *B. pseudomallei* stationary-phase cultures by using HPLC coupled with tandem MS, an approach which allowed for more accurate and quantitative identification of the AHLs present. By analysing extracts from stationary-phase cultures of null mutants in each of the three AHL synthase genes, as well as from *Escherichia coli* strains individually expressing the three AHL synthase genes, we identified the major products of $BpsI_2$ and $BpsI_3$. We then compared the biofilm formed by each of these AHL synthase mutants with that formed by wild-type strain KHW. Our results demonstrate a critical role played by the BpsI–BpsR QS system in regulating biofilm formation in *B. pseudomallei*.

METHODS

Strains, media and culture conditions. Bacterial strains and plasmids used are described in Table 1. All cultures were routinely grown in Luria–Bertani (LB) medium (Becton Dickinson) at 37 °C, with shaking at 100 r.p.m., unless otherwise mentioned. Where appropriate, antibiotics for *E. coli* cultures were added at the following final concentrations: trimethoprim, 40 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; kanamycin, 40 μg ml^{-1}. For *B. pseudomallei* cultures, final concentrations of antibiotics used were: trimethoprim, 40 μg ml^{-1}; tetracycline, 50 μg ml^{-1}; kanamycin, 200 μg ml^{-1}; gentamicin, 100 μg ml^{-1}.

Construction of plasmids. *B. pseudomallei* DNA used in the constructs was obtained by amplifying genomic DNA from isolate KHW by using PCR and DNA polymerase (Biotools). Restriction enzymes and T4 DNA ligase were purchased from Promega. The primers used for PCR amplification and DNA construction are listed in Supplementary Table S1 (available with the online version of this paper), and gene-specific primers used for quantitative real-time PCR (qRT-PCR) experiments are listed in Supplementary Table S2 (available with the online version of this paper). Oligonucleotides were purchased from 1st Base.

For heterologous expression of *B. pseudomallei* AHL synthase genes in *E. coli* DH5α, DNA fragments containing the entire coding region of *bpsI* (660 bp), $bpsI_2$ (757 bp) and $bpsI_3$ (606 bp) were amplified by PCR with the primer pairs BpsI (*Eco*RI)F/BpsI (*Pst*I)R, $BpsI_2$F

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristic	Reference or source
***B. pseudomallei* strains**		
KHW	Virulent clinical isolate from Singapore	Song *et al.* (2005)
K96243	Clinical isolate from Thailand	Holden *et al.* (2004)
H11	Virulent clinical isolate; Gen^r Chl^r	E. H. Yap
KHW *bpsI*::Km	KHW derivative; *bpsI* disrupted by Km^r cassette	Song *et al.* (2005)
KHW $bpsI_2$::Tet	KHW derivative; $bpsI_2$ disrupted by Tet^r cassette	This study
KHW $bpsI_3$::Tet	KHW derivative; $bpsI_3$ disrupted by Tet^r cassette	This study
KHW *bpsR*::Km	KHW derivative; *bpsR* disrupted by Km^r cassette	Song *et al.* (2005)
***E. coli* strains**		
DH5α	F^- φ80*lacZ*αM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_k^- m_k^+) *pho*A *supE44 thi*-1 *gyrA96 relA1 tonA*	Invitrogen
Sm10λpir	Mobilizing strain	Simon *et al.* (1983)
Plasmids		
pFTC1	Tetracycline resistance FRT vector, Tet^r	Choi *et al.* (2005)
pMLBAD	Arabinose inducible expression vector, pBBR1 *ori*, Tmp^r	Lefebre & Valvano (2002)
pCYY	Mini-CTX1 carrying promoter less *lacZYA* cassette from pMC1403, Tet^r	Chan & Chua (2005)
pK18*mobsacB*	*oriT*, *sacB*, Km^r	Schäfer *et al.* (1994)
pML-I	pMLBAD ligated to *bpsI* gene	This study
pML-I_2	pMLBAD ligated to $bpsI_2$ promoter region and ORF	This study
pML-I_3	pMLBAD ligated to $bpsI_3$ ORF	This study
pML-R_3	pMLBAD ligated to $bpsR_3$ ORF	This study
pRI′-lacZ	*bpsR* promoter and ORF, and *bpsI* promoter region ligated to *lacZ* gene on pCYY	Song *et al.* (2005)
pR_2-lacZ	$bpsR_2$ promoter region and ORF cloned into pCYY	This study
pR_2I_2′-lacZ	$bpsI_2$ promoter region ligated to *lacZ* gene on pR_2-lacZ	This study
pI′-lacZ	*bpsI* promoter region ligated to *lacZ* gene on pCYY	This study
pI_3′-lacZ	491 bp upstream of $bpsI_3$ start site ligated to *lacZ* gene on pCYY	This study
pK18I_2::Tet	pK18 carrying $bpsI_2$ ORF disrupted by 2.1 kb tetracycline resistance cassette	This study

(*Eco*RI)/BpsI_2R (*Pst*I) and BpsI_3F (*Eco*RI)/BpsI_3R (*Pst*I), respectively. The $bpsI_2$ fragment also included 88 bp upstream of the region containing a putative $bpsI_2$ promoter. The restriction enzyme-digested products were then ligated into the respective sites on pMLBAD to obtain pML-I, pML-I_2 and pML-I_3, respectively. The constructs were introduced into *E. coli* DH5α by electroporation and selected on LB agar containing trimethoprim.

Reporter *lacZ* translational fusion plasmids were constructed as follows: pR_2I_2′-lacZ was constructed by amplifying the $bpsR_2$ ORF by using the primer pair BpsR_2 (*Spe*I)F and BpsR_2 (*Eco*RI)R, and ligating the *Spe*I–*Eco*RI-digested PCR product with pCYY to obtain pR_2-lacZ. Next, a 423 bp fragment including the predicted promoter and 5′ region of the $bpsI_2$ gene was amplified by using the primer pair BpsI_2Pro (*Eco*RI)F and BpsI_2Pro$_2$ (*Bam*HI)R, and inserted into *Eco*RI–*Bam*HI-digested pR_2-lacZ to give pR_2I_2′-lacZ. pI_3′-lacZ was constructed by amplifying a 635 bp DNA fragment comprising 144 bp of the 5′ end of the $bpsI_3$ and the upstream promoter region by using the primer pair BpsI_3 5′ (*Pst*I)F2 and BpsI_3 5′ (*Eco*RI)R2. The PCR product was digested with *Pst*I and *Eco*RI and inserted into pCYY. pI′-lacZ was constructed by amplifying a 490 bp DNA fragment comprising 255 bp of the 5′ end of the *bpsI* gene and the upstream promoter region by using the primers BpsI 5′ (*Spe*I)F and BpsI 5′ (*Eco*RI)R. The PCR product was doubly digested with *Spe*I and *Eco*RI and inserted into pCYY. pML-R_3 was created by amplifying the $bpsR_3$ ORF by using the primer pair BpsR3(*Eco*RI)F and BpsR3(*Xba*I)R, and inserting the *Eco*RI–*Xba*I-digested PCR product into pMLBAD. The resulting plasmid pML-R_3 was then introduced into DH5α/pI_3′-lacZ and DH5α/pI′-lacZ when investigating whether BpsR_3 could respond to AHLs to activate transcription of $bpsI_3$ and *bpsI*, respectively.

Construction of *B. pseudomallei* mutants. The $bpsI_2$ and $bpsI_3$ genes were amplified with the primer pairs BpsI_2F (*Eco*RI)/BpsI_2R (*Pst*I) and BpsI_3F (*Eco*RI)/BpsI_3R (*Pst*I) as above, and cloned into the suicide vector, pK18mobsacB. A 2.1 kb tetracycline resistance cassette was excised from pFTC1 and inserted into the *Sal*I site on $bpsI_2$ (+115 bp of the predicted ORF) and $bpsI_3$ (+159 bp of the predicted ORF) to create the plasmids pKI_2::Tet and pKI_3::Tet, respectively. The plasmids were transferred into *E. coli* Sm10λpir by electroporation and then mobilized into *B. pseudomallei* by conjugation. Transconjugants were first selected for resistance to tetracycline and then for resistance to 10 % sucrose. Genomic DNA was extracted from the mutants to confirm, via PCR, the disruption of the $bpsI_2$ and $bpsI_3$ genes, respectively.

Extraction of AHLs. *B. pseudomallei* was cultured in 10 ml LB medium at 37 °C with shaking at 100 r.p.m. AHLs were extracted from the supernatant of early exponential-phase (OD_{600}=0.5, ~3 h) or stationary-phase (OD_{600}=2.0, ~24 h) cultures. After removing most of the cells by centrifuging at 4000 r.p.m. for 15 min, the supernatant was filtered through a 0.2 μM polyethersulfone Minisart filter (Sartorius). The internal standard *N*-hexanoyl-L-homoserine lactone-d_3 (d_3-C6HL, final concentration 100 nM; Cayman Chemicals) and 1 ml 1 M hydrochloric acid was added to the filtrate and left at room temperature for 16 h. Sample acidification recircularizes the open ring form of hydrolysed AHLs generated under alkaline conditions during bacterial growth and therefore improves extraction yield (Cataldi *et al.*, 2009; Yates *et al.*, 2002). The acidified supernatant samples were extracted twice with equal volumes of ethyl acetate and dried by using a rotary evaporator before resuspending in 250 μl acetonitrile (i.e. a concentration factor of 40). *E. coli* DH5α harbouring pBDI, pBDI_2 or pBDI_3 was cultured in 10 ml LB medium containing trimethoprim and 0.2 %

arabinose. AHLs were extracted from cell-free supernatants of mid-exponential-phase cultures (OD_{600}=0.8–1.0), as described above. All extractions were carried out in triplicate.

HPLC-MS/MS analysis of AHLs. Analysis of AHLs was carried out by using an Accela HPLC system coupled to an LTQ Orbitrap XL hybrid Fourier Transform Mass Spectrometer (Thermo Fisher Scientific). Twenty microlitres of each extract was injected onto a 1.9 μm particle size, 50 mm × 2.1 mm Hypersil Gold C18 RP-HPLC column (Thermo Fisher Scientific). HPLC conditions were as follows: mobile phase A (water mobile phase acidified with 0.1 % acetic acid), B (acetonitrile with 0.1 % acetic acid); flow rate 130 μl min^{-1}; 5 % B for 3 min, then linearly switched to 95 % B over 7 min and maintained for 5 min, and then linearly changed back to 5 % B in 2 min and maintained for 8 min. The eluate from the RP-HPLC column was introduced into the mass spectrometer during the first 20 min of the run. Initially, we carried out select reaction monitoring (SRM) experiments by using synthetic C8HL to optimize the ionization energies for maximum peak intensity. Next, 100 nM samples of synthetic d_3-C6HL, *N*-hexanoylhomoserine lactone (C6HL), C8HL, C10HL, OHC8HL, OHC10HL, OHC12HL, oxoC8HL, *N*-(3-oxodecanoyl)homoserine lactone and *N*-(3-oxododecanoyl)homoserine lactone were analysed via SRM to confirm that these compounds could be separated and detected under selected experimental conditions. With the exception of d_3-C6HL, OHC8HL, OHC10HL and OHC12HL (gifts from Otsuka Pharmaceuticals), the other synthetic standards were purchased from Sigma Aldrich. We then analysed the supernatant extracts using multiple MS scanning modes. First, the Fourier transform function was used to determine accurately the mass of all compounds present in the extract, with the HPLC elution profile being the same as that for the SRM of standards. We then searched mass peaks corresponding to AHLs from C4 to C16, and their hydroxy- and oxo- versions, and where the peak elution times were reconcilable with those of the standards we used.

SRM experiments were then performed in positive-ion mode with Q1 scanning for parent ions of putative AHL species identified in the first round of screening, and Q3 scanning for *m*/*z* 102.1 (Gould *et al.*, 2006). The instrument parameters were as follows: ion spray voltage of 3500 V, collision energy of 40 V, capillary temperature of 275 °C; nitrogen was used as the collision gas. Elution times of AHL species were compared with those of standards whenever possible. AHL species that had been positively identified as being present via SRM were then subjected to a product ion scan, and the fragmentation patterns were compared with those of the standards. This provided another layer of verification on AHL identity. For AHL quantification, the integrated peak areas of detected AHL species obtained via the extracted ion chromatogram from SRM experiments were compared with that of the d_3-C6HL internal standard peak area for that same sample by using the equipment software.

qRT-PCR. Total RNA was extracted in triplicate from *B. pseudomallei* cultures at OD_{600} 0.5, 1 and 2, by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Synthesis of cDNA and qRT-PCR were performed as described by Lee *et al.* (2010).

***β*-Galactosidase assays.** Overnight cultures of DH5α/pRI′-lacZ, DH5α/pR_2I_2′-lacZ and DH5α/pR_3I′-lacZ were diluted (1 : 50) in fresh LB broth containing the relevant antibiotic(s) and incubated at 37 °C with shaking at 100 r.p.m. until OD_{600}=0.2, when AHLs were added at the relevant final concentrations. β-Galactosidase activity was determined after 2 h (OD_{600}=0.6–0.8) according to the protocol described by Miller (1972). For bacteria harbouring pML-R_3, 0.2 % arabinose was also added to the culture medium to induce expression of *bpsR*$_3$. For the reporter strains DH5α/pI′lacZ (pML-R_3) and DH5α/pI_3′lacZ (pML-R_3) the incubation period in the presence of AHLs was extended to 16 h as there was no significant induction of β-galactosidase expression after 2 h, even in the presence of 1 μM AHLs. Exogenous AHLs used in the assays were C8HL, C10HL, OHC12HL, OHC8HL and OHC10HL. All AHL stocks were solubilized in 70 % acetonitrile/water acidified with 0.1 M HCl final concentration. All results were analysed by using Student's *t*-test.

Biofilm assays. Biofilm formation on polystyrene wells after incubation for 24 or 48 h was assayed as described by Taweechaisupapong *et al.* (2005). Briefly, overnight cultures in LB medium were adjusted to OD_{600}=0.8 and 200 μl was added into each of six replicate wells of a flat-bottomed 96-well PVC microtitre plate (Nunclon). After 3 h static incubation at 37 °C to facilitate adhesion of bacteria, the cultures were aspirated, and fresh medium was added. After a further 21 h of incubation, the wells were washed with sterile distilled water and replaced with fresh LB broth media. After a further 24 h of incubation, the 2-day-old biofilm was washed in distilled water, fixed in methanol, stained with 1 % crystal violet and solubilized in 33 % (v/v) glacial acetic acid. Biofilm formation was quantified by measuring OD_{590}. For quantification of biofilm formed after 24 h, the addition of LB broth media after 24 h of incubation was omitted, and the biofilm was fixed at that stage. For assays involving exogenously added AHLs, overnight cultures were diluted 1 : 20 in fresh medium supplemented with AHLs and grown for a further 12–15 h, and the biofilm assay was then repeated as above, each time adding fresh LB medium containing the appropriate concentration of AHLs. All results were analysed by using Student's *t*-test.

RESULTS

Qualitative and quantitative evaluation of AHLs produced by *B. pseudomallei* by using tandem MS

AHLs were extracted and analysed from the culture supernatants of *B. pseudomallei* KHW, H11 and K96243 during the stationary growth phase, when the concentrations of such signalling molecules were expected to be at their highest. Spiking the culture supernatants with a known quantity of a d_3-C6HSL internal standard also allowed us to quantify the AHLs detected. All positively identified species were compared with the elution times and fragmentation patterns of synthetic equivalents, which, along with the inherent specificity of SRM, greatly reduces the chances for misidentification of AHLs. Only three AHL species, C8HL, OHC8HL and OHC10HL, were detected in the stationary-phase culture supernatants of *B. pseudomallei* KHW (Table 2). A comparison of the AHLs produced by KHW, H11 and K96243 revealed that both KHW and K96243 produced the same three species of AHLs although H11 also produced minor quantities of OHC12HL and C10HL, comprising <0.5 % of the total AHLs produced (Table 2). C8HL was the predominant AHL produced by each of the three *B. pseudomallei* strains, accounting for >95 % of the total AHLs produced (Table 2).

Growth-related changes in expression of AHL synthases and AHL production

Transcript levels of each of the three LuxI-LuxR homologues were quantified during early exponential phase (OD_{600}~0.5), late exponential phase (OD_{600}~1.0) and stationary phase (OD_{600}~2.0). Expression of both *bpsI*

Table 2. Comparison of AHLs produced by different *B. pseudomallei* isolates and AHL synthase mutants derived from KHW

Data are shown as mean ± SD. Figures in parentheses refer to percentage of each AHL species relative to total AHLs produced by the respective strain. ND, Not detected.

AHL	Extracellular AHLs produced (nM)					
	KHW	H11	K96243	KHW*bpsI*	KHW*bpsI*$_2$	KHW*bpsI*$_3$
C8HL	1703 ± 126 (96.1)	10 846 ± 1572 (95.3)	2779 ± 686 (95.6)	ND	714 ± 163	1320 ± 108
OHC8HL	31 ± 8 (1.7)	18 ± 4 (0.2)	100 ± 10 (3.4)	ND	19 ± 7	ND
OHC10HL	39 ± 13 (2.2)	439 ± 106 (3.9)	29 ± 7 (1)	11 ± 2	ND	8 ± 2
OHC12HL	ND	25 ± 5 (0.2)	ND	ND	ND	ND
C10HL	ND	48 ± 11 (0.4)	ND	ND	ND	ND

and *bpsR* was upregulated during stationary phase, as reported previously (Song *et al.*, 2005). Expression of both *bspI* and *bpsR* during stationary phase was about 10-fold higher than the early exponential phase (Fig. 1). In contrast, expression of both *bpsI*$_2$ and *bpsR*$_2$ during stationary phase was downregulated by approximately 200- and 10-fold, respectively, when compared with the early exponential phase (Fig. 1). Expression of *bpsI*$_3$ was only weak throughout the growth phases, while its cognate receptor, BpsR$_3$, was highly expressed at all growth phases (Fig. 1).

As not all AHL synthases were maximally transcribed during the stationary phase, we compared the amounts of the three major species of AHLs produced by *B. pseudomallei* during early exponential and stationary phases (Table 3). During early exponential phase, OHC10HL was the major product detected, and was three times more abundant than C8HL. Total AHL produced by *B. pseudomallei* KHW increased from 184 nM during early exponential phase to 1774 nM during stationary phase, but the amount of OHC10HL produced actually decreased 3.5-fold from 138 nM during early exponential phase to 39 nM during stationary phase (Table 3). This agrees with the decrease in *bpsI*$_2$ transcript levels during stationary phase and also links BpsI$_2$ to the synthesis of OHC10HL (Fig. 1a).

AHL products synthesized by BpsI$_2$ and BpsI$_3$

AHL synthase null mutants were created by insertional inactivation of *bpsI*, *bpsI*$_2$ and *bpsI*$_3$. When AHLs extracted from stationary-phase culture supernatants of each mutant were profiled and compared with those of wild-type KHW, we found that C8HL and OHC8HL were absent from the supernatant of the *bpsI* mutant, while OHC10HL and OHC8HL were absent in the supernatant of the *bpsI*$_2$ and *bpsI*$_3$ mutants, respectively (Table 2). While *bpsI*$_3$ was weakly transcribed in wild-type cells during stationary phase, there was no *bpsI*$_3$ transcript in the *bpsI* mutant (Fig. 1a, data not shown). This suggests that BpsI (or its product) is required for the transcription of *bpsI*$_3$ albeit via an unknown mechanism, leading to an absence of OHC8HL production in the *bpsI* mutant.

We also cloned *bpsI*, *bpsI*$_2$ and *bpsI*$_3$ separately into the arabinose-inducible vector, pMLBAD, for heterologous expression of each AHL synthase gene in *E. coli* DH5α. DH5α/pML-I produced C8HL as the major product (>99 % of total AHLs in the culture supernatant), in

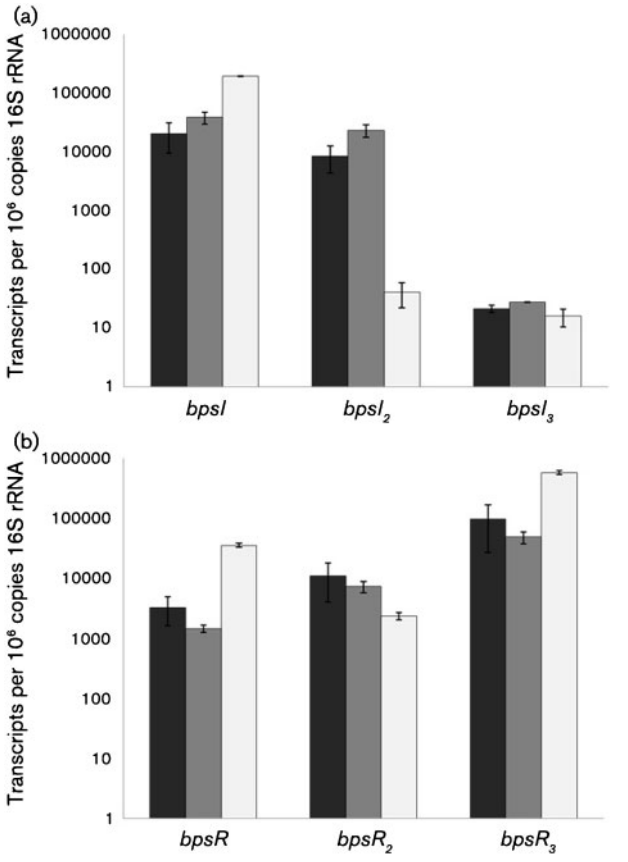

Fig. 1. qRT-PCR analysis of growth-related changes in AHL synthase expression in *B. pseudomallei* KHW. (a) Transcript levels of the AHL synthase genes, *bpsI*, *bpsI*$_2$ and *bpsI*$_3$, at mid-exponential (OD$_{600}$=0.5, black bars), late exponential (OD$_{600}$=1.0, grey bars) and stationary (OD$_{600}$=2.0, white bars) phase. (b) Transcript levels of each transcriptional regulator of the AHL synthases, *bpsR*, *bpsR*$_2$ and *bpsR*$_3$, at mid-exponential (OD$_{600}$=0.5, black bars), late exponential (OD$_{600}$=1.0, grey bars) and stationary (OD$_{600}$=2.0, white bars) phase. Error bars, SD from three experiments.

Table 3. Comparison of AHLs detected in supernatant extracts of *B. pseudomallei* KHW cultures at early exponential and stationary phases of growth

Data are shown as mean ± SD. ND, Not detected.

AHL	Extracellular AHL concentration (nM)	
	Early exponential phase	Stationary phase
C8HL	46 ± 8	1703 ± 126
OHC8HL	ND	31 ± 8
OHC10HL	138 ± 6	39 ± 13

agreement with our earlier identification (Table 4) (Song *et al.*, 2005). In addition to C8HL, minor amounts of C6HL, C10HL, OHC8HL and OHC10HL were detected, but together these accounted for <1 % of the total AHLs synthesized. DH5α/pML-I_2 produced predominantly OHC10HL (>99 % of the total AHLs in the culture supernatant; Table 4). Small quantities of other 3-hydroxy-substituted AHLs, such as OHC8HL (<0.1 %) and OHC12HL (0.6 %) were also detected. Together with the AHL profile of a *bpsI*$_2$ mutant, this confirmed that $BpsI_2$ is responsible for the production of mainly OHC10HL in *B. pseudomallei*. DH5α overexpressing *bpsI*$_3$ produced mainly OHC8HL, accounting for 80 % of the total AHLs produced (Table 4). A small amount of OHC10HL was also produced when *bpsI*$_3$ was overexpressed in DH5α but when considered together with the absence of only OHC8HL in the culture supernatant of the *bpsI*$_3$ mutant, we may conclude that $BpsI_3$ is responsible for OHC8HL synthesis in *B. pseudomallei* (Table 2).

Response of BpsR, $BpsR_2$ and $BpsR_3$ to cognate AHLs

Having confirmed the AHLs produced by each AHL synthase, we then compared the responsiveness of each of the LuxR homologues to the AHLs produced by *B. pseudomallei*. Using the reporter strain DH5α/pRI′-lacZ, we confirmed earlier reports that BpsR responded most strongly towards C8HL, and less so towards C10HL as a ligand for transcriptional activation of *bpsI*. BpsR could also activate the expression of *bpsI* weakly in the presence of OHC8HL, but was unable to do so with OHC10HL or OHC12HL, suggesting that BpsR has a higher affinity for unsubstituted AHLs and a much lower affinity for 3-hydroxy-substituted AHLs (Fig. 2a). Conversely, $BpsR_2$ has a higher affinity for 3-hydroxy-substituted AHLs as compared with unsubstituted AHLs in its transcriptional regulation of *bpsI*$_2$ expression (Fig. 2b). The affinity of $BpsR_2$ towards 3-hydroxy-substituted AHLs was OHC10HL>OHC12HL>OHC8HL. Thus, $BpsR_2$ is capable of positively auto-regulating the transcription of *bpsI*$_2$ for synthesis of OHC10HL, its cognate AHL ligand. When we tested the ability of $BpsR_3$ to activate transcription of the *bpsI*$_3$ promoter–*lacZ* fusion (pI$_3$′-lacZ), we did not detect any significant difference in β-galactosidase activity between the uninduced culture and those with exogenous AHLs up to 1 mM final concentrations (data not shown). To show that $BpsR_3$ was functional, we then tested its ability to activate transcription of *bpsI-lacZ* (pI′-lacZ) in the presence of AHLs produced by *B. pseudomallei* KHW. $BpsR_3$ was capable of activating the expression of *bpsI*, albeit weakly, and only in the presence of high concentrations of exogenous AHLs (500 nM) and requiring longer exposure to the lactones (16 h instead of 2 h) (Fig. 2c). Exogenously added OHC8HL, OHC10HL and OHC12HL were all capable of causing a significant increase in reporter gene expression, although note that OHC8HL, the major product of its cognate AHL synthase, elicited the strongest response from $BpsR_3$.

QS control of biofilm formation

We then investigated whether biofilm formation, a multicellular behaviour coordinated by QS in many bacterial species, was similarly regulated in *B. pseudomallei*, and if so, which of the three QS systems played a major role in the process. We first compared the amount of biofilm formed on polystyrene by static cultures of wild-type *B. pseudomallei*

Table 4. AHL species produced by recombinant *E. coli* DH5α expressing each of the AHL synthase genes, *bpsI*, *bpsI*$_2$ and *bpsI*$_3$

Data are shown as mean ± SD. Figures in parentheses refer to percentage of each AHL species relative to total AHLs produced. ND, Not detected.

AHL	Extracellular AHL concentration (nM)		
	DH5α/pML-I	DH5α/pML-I_2	DH5α/pML-I_3
C6HL	68 ± 31 (<0.1)	ND	ND
C8HL	22 3042 ± 18 359 (99.4)	ND	ND
C10HL	1098 ± 216 (0.5)	ND	ND
OHC8HL	56 ± 5 (<0.1)	7 ± 1 (<0.1)	28 ± 4 (80)
OHC10HL	81 ± 44 (<0.1)	11 401 ± 1895 (99.3)	7 ± 2 (20)
OHC12HL	ND	68 ± 6 (0.6)	ND

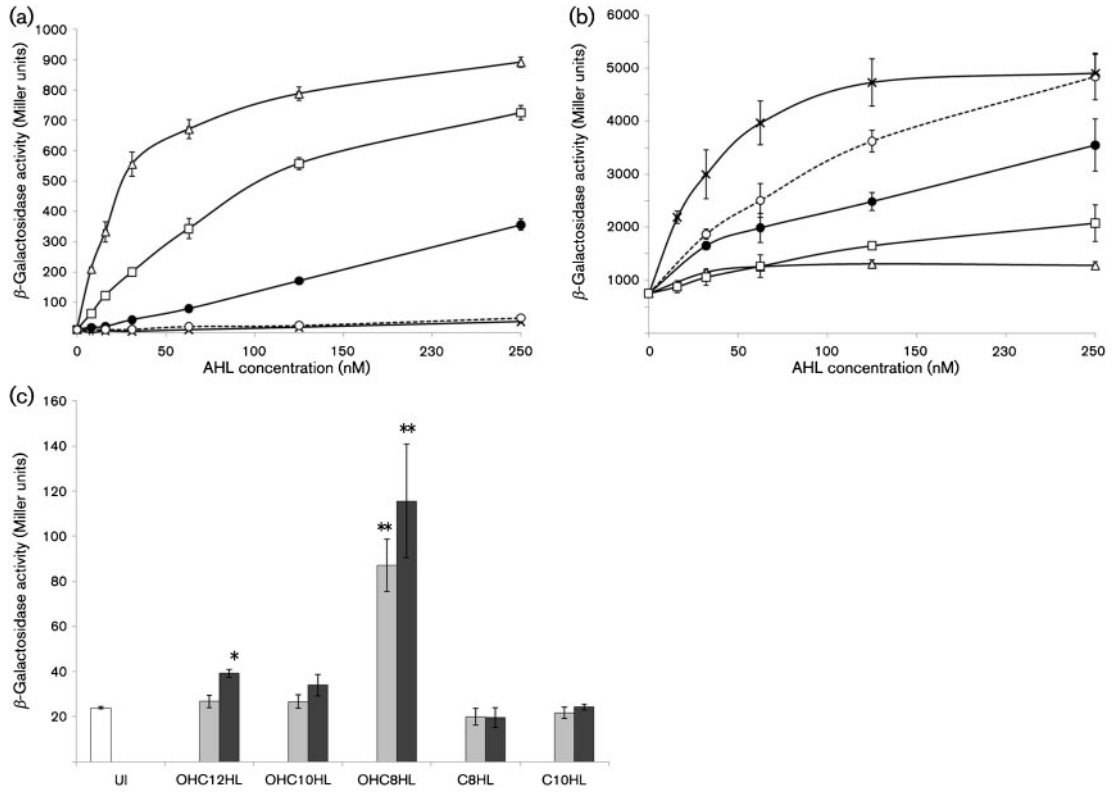

Fig. 2. Comparison of BpsR-, $BpsR_2$- and $BpsR_3$-regulated AHL synthase expression in response to exogenous AHLs. (a, b) β-Galactosidase activities of DH5α/pRI′-lacZ (a) and DH5α/pR_2I_2′-lacZ (b) in the presence of increasing concentrations of exogenous AHLs. △, C8HL; □, C10HL; ●, OHC8HL; ×, OHC10HL; ○, OHC12HL. (c) β-Galactosidase activity of DH5α/pI′-lacZ (pML-R3) in the absence of exogenous AHLs (white bar), and in the presence of 500 nM (light grey bars) or 1 μM (black bars) exogenous AHLs. Error bars represent standard deviations of three experiments. Asterisks denote statistically significant differences compared with the uninduced sample; *$P<0.05$ and **$P<0.01$.

KHW and each of the AHL synthase mutants after 24 and 48 h. Biofilm formation was most severely impaired in the *bpsI* null mutant (~75 % reduction after 48 h) when compared with the wild-type (Fig. 3a). The $bpsI_3$ mutant also produced less biofilm (~40 % that of the wild-type) albeit not as severely impaired as the *bpsI* mutant, but the $bpsI_2$ mutant was not impaired in biofilm formation. A similar trend was observed upon crystal violet staining of a 24 h biofilm, where both the *bpsI* and $bpsI_3$ mutants formed significantly reduced biofilms (Fig. 3a). Since BpsR is the cognate receptor for C8HL, it is not surprising that the *bpsR* null mutant was severely impaired in biofilm formation after 48 h incubation, showing a ~60 % reduction compared with the wild-type (Fig. 3a).

Since we had determined the nature and abundance of AHL signals present in stationary-phase cultures of *B. pseudomallei*, we used exogenous C8HL, the major product of BpsI, to restore biofilm formation in the *bpsI* mutant. A dose-dependent increase in biofilm formation in the *bpsI* mutant occurred in the presence of 1 and 2 μM exogenous C8HL (Fig. 3b). No increase in biofilm formation by the *bpsI* mutant was obtained by adding 1 μM exogenous OHC8HL and OHC10HL, although a modest increase was obtained when these AHLs were added at 2 μM (Fig. 3b). This agrees with the data showing that BpsR has a much higher affinity for unsubstituted AHLs than 3-hydroxy-substituted AHLs (Fig. 2a).

Next, we tested if the reduction in biofilm formed by the $bpsI_3$ mutant could be restored to wild-type levels by exogenous OHC8HL. Surprisingly, exogenous addition of OHC8HL could not restore biofilm formation in the $bpsI_3$ mutant (Fig. 3c). Instead, a further reduction in biofilm formation was observed when OHC8HL was added at concentrations several-fold above that detected in stationary-phase cultures, i.e. at 200 nM and 1 μM (Fig. 3c). This suggests that the reduction in biofilm formation in the $bpsI_3$ mutant is probably due to a mechanism that is independent of OHC8HL per se. Addition of 1 μM C8HL increased biofilm formation in a $bpsI_3$ mutant slightly ($P<0.05$), while addition of 1 μM OHC10HL yielded a reduction in biofilm formation similar to that caused by OHC8HL (Fig. 3c). The antagonistic property of OHC8HL

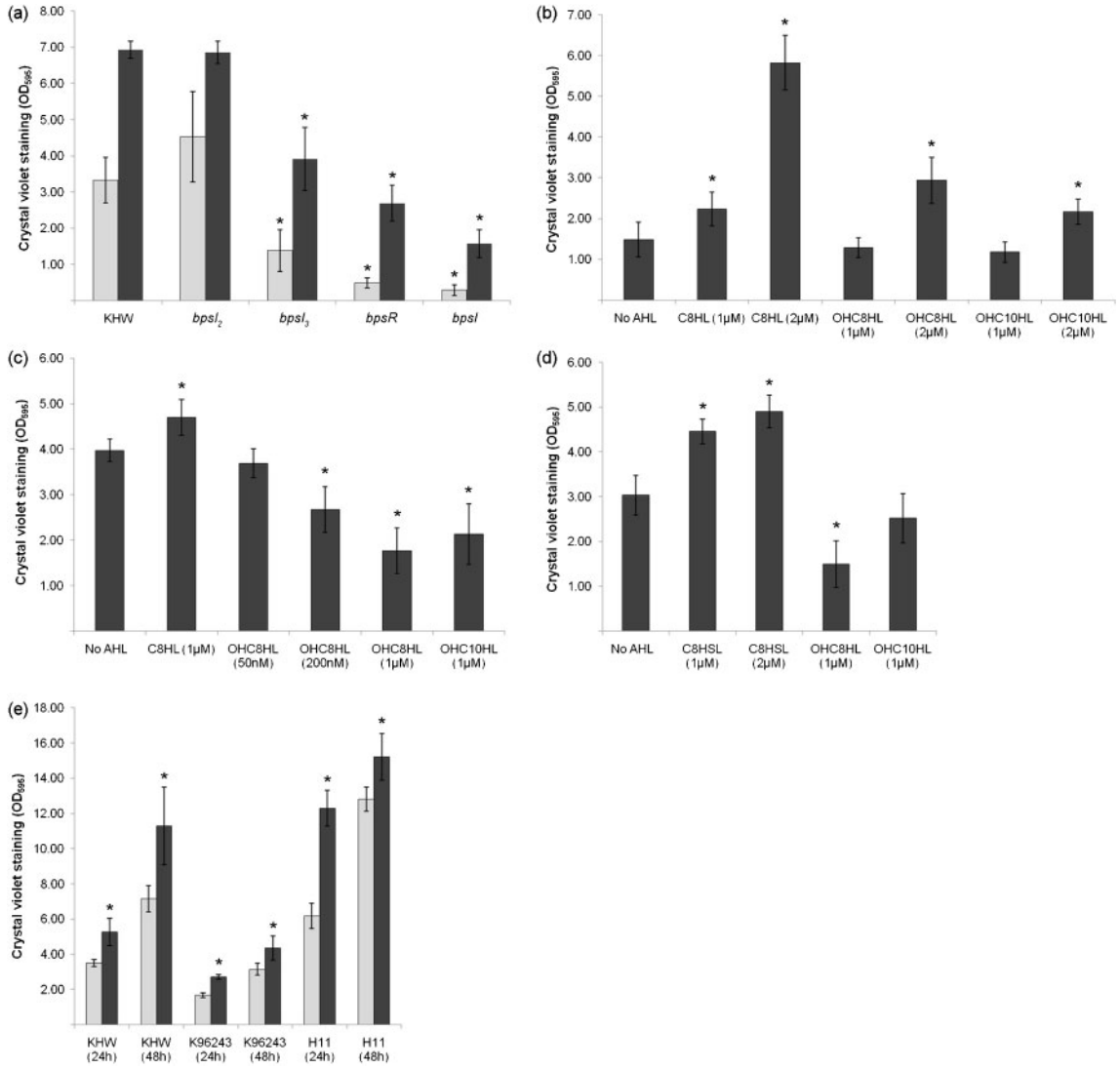

Fig. 3. QS control of biofilm formation in *B. pseudomallei* KHW. (a) Comparison of biofilm formation by *B. pseudomallei* KHW and its isogenic *bpsI*, *bpsI*$_2$ and *bpsI*$_3$ null mutants in 24 h (grey bars) and 48 h (black bars) static cultures. Absence of *bpsI* reduced biofilm formation to 9 and 23 % of wild-type levels after 24 and 48 h static culture, respectively. There was no reduction in biofilm formation in the *bpsI*$_2$ mutant and a smaller, but significant, reduction in the *bpsI*$_3$ mutant to 42 and 56 % of wild-type levels after 24 and 48 h static culture, respectively. (b) Restoration of biofilm formation by the *bpsI* mutant to almost wild-type KHW level in the presence of 2 μM exogenous C8HL and partial restoration in the presence of either 1 μM exogenous C8HL, 2 μM exogenous OHC8HL or 2 μM exogenous OHC12HL. Asterisks (in a and b) denote statistically significant differences compared with biofilm formation by wild-type KHW; *P<0.05. (c) Dose-dependent reduction in biofilm formation by the *bpsI*$_3$ mutant after 48 h in the presence of exogenous OHC8HL (ranging from 50 nM to 1 μM) and partial restoration in the presence of 1 μM exogenous C8HL. (d) Opposing effects of exogenous C8HL and OHC8HL in enhancement and suppression of biofilm formation, respectively, in 24 h static cultures of wild-type KHW. (e) Enhancement of biofilm formation after 24 and 48 h in different *B. pseudomallei* strains in the presence of 2 μM exogenous C8HL. Grey and black bars represent biofilm formation in the absence and presence of 2 μM exogenous C8HL, respectively. Asterisks (in c–e) denote statistically significant difference compared with biofilm formation in the absence of exogenous AHLs; *P<0.05. Error bars, SD of six experiments.

on biofilm formation was also observed in wild-type KHW, in which a 50 % reduction was observed in the presence of 1 μM exogenous OHC8HL compared with only a 17 % reduction in the presence of 1 μM OHC10HL (Fig. 3d).

To investigate whether C8HL positively regulates biofilm formation in different *B. pseudomallei* strains, we measured the 24 and 48 h biofilm growth of strains KHW, K96243 and H11 after 2 μM C8HL supplementation. There was a large

variation in biofilm formation across the strains; for example, strain K96243 formed a weak biofilm at both time points measured, while H11 formed a remarkably dense biofilm even after 24 h of static culture (Fig. 3e). Nevertheless, biofilm formation could be further improved in each strain by the exogenous addition of 2 μM C8HL (Fig. 3e).

DISCUSSION

Analysis of AHLs using LC/MS/MS allows for the sensitive detection of AHLs extracted from complex media without any requirement for prior purification. In the first round of HPLC/MS screening, AHLs were detected by using a high-resolution Fourier transform MS set-up. Adapted from the method described by Cataldi *et al.* (2009), this round of screening provides a method for the systematic and unbiased detection of the AHLs present, and identifies putative AHLs for a more accurate subsequent analysis. The second round of HPLC/MS/MS screening, involving retention times and SRM, provides a means to verify the putative AHLs by observing the presence of a product ion of 102.1 *m/z*, corresponding to the lactone ring (Gould *et al.*, 2006). Previous attempts to characterize AHLs produced by *B. pseudomallei* had relied on matching the elution times of unknown AHLs in extracts with the elution times of known AHLs ascertained by using a reporter strain and bioassay (Song *et al.*, 2005; Valade *et al.*, 2004). By using such an approach, without any prior knowledge on the nature and abundance of the AHL species present in the extract, assigning peaks to the wrong synthetic standard is possible. Another drawback is that the reporter strain used in the bioassay is unlikely to be equally sensitive to all the different AHLs present in the extracts, so the method cannot be quantitative.

Although there is a report in which tandem MS was employed for the detection of AHLs in *B. pseudomallei*, it is unclear why the results presented were different from our own (Ulrich *et al.*, 2004b). Precursor ion scanning was employed as the sole mode of AHL detection, which raises the possibility of false positive AHL species identification, especially as analysis was performed on crude extracts of culture supernatants, and in the absence of verification by other MS methods. There are two lines of evidence suggesting that the actual AHL profile of *B. pseudomallei* described herein is more accurate. One is the agreement we observed across different experiments. For instance, all the AHLs produced by *B. pseudomallei* KHW could be accounted for from extracts of *E. coli* which expressed individual AHL synthase genes. The product(s) of each AHL synthase as deduced from the AHLs present or absent in the extracts of each of the AHL synthase mutants also agreed with the data from heterologous expression of the AHL synthases. Furthermore, the growth-related changes in abundance of the types of AHLs produced could be explained from the transcript levels of the respective AHL synthases at different growth phases. The second line of evidence is that our data match closely the AHLs produced by two other *Burkholderia* species, namely *Burkholderia thailendensis* and *Burkholderia mallei* (Chandler *et al.*, 2009; Duerkop *et al.*, 2007, 2008). The *B. mallei* BmaI1, a homologue of BpsI, also produced C8HL while BmaI3, a homologue of $BpsI_3$, produced OHC8HL (Chandler *et al.*, 2009; Duerkop *et al.*, 2008). Similarly, in *B. thailendensis*, BtaI1, BtaI2 and BtaI3 are responsible for the production of C8HL, OHC10HL and OHC8HL, respectively (Chandler *et al.*, 2009). Difficulties in elucidating the AHL structure would also explain the previous conflicting description of AHL products of *B. mallei* and *B. thailendensis*, as both species were reported to produce C6HL, C8HL and C10HL (Ulrich *et al.*, 2004a, b).

In many species of bacteria, QS is involved in co-coordinating the process of biofilm development and differentiation (Davies *et al.*, 1998; Kirisits & Parsek, 2006). Based on our results, we hypothesize that in *B. pseudomallei*, C8HL produced by BpsI synthase activates its cognate receptor, BpsR, which then transcribes target genes required for the formation of a mature biofilm. We also show that despite significant heterogeneity in the amount of biofilm formed by different *B. pseudomallei* strains, biofilm formation can be further induced by the addition of exogenous C8HL, suggesting a conservation in this positive regulatory mechanism across different strains. Hence, AHL analogues that antagonize C8HL-mediated activation of BpsR would be ideal candidates for prevention or reduction of biofilm formation by *B. pseudomallei*. Such compounds could also deliver a synergistic effect on improving antimicrobial susceptibility of biofilm bacteria.

It is interesting to note that exogenous OHC8HL and OHC10HL could partially restore biofilm formation when C8HL was absent (in a *bpsI* mutant), yet similar concentrations of these AHLs disrupted biofilm formation when C8HL was present (in the wild-type and $bpsI_3$ mutant) (Fig. 3b, d). One explanation of the above observations is that both OHC8HL and OHC10HL become partial antagonists of BpsR at the concentrations tested and compete with endogenous C8HL for binding to BpsR. It is also possible that these exogenous 3-hydroxy-substituted AHLs activate either $BpsR_2$ or $BpsR_3$, which in turn affects target gene expression in a manner that is opposite to that of BpsR. For example, the CepIR and CciIR QS systems in *Burkholderia cepacia* co-regulate 197 ORFs, of which the majority (~85 %) are positively regulated by CepR and negatively regulated by CciIR (O'Grady *et al.*, 2009).

Our results demonstrate that C8HL, produced by BpsI, is the major AHL species present in stationary-phase cultures of *B. pseudomallei* under our experimental conditions. However, it is possible that under different growth conditions, such as culture at 25 °C, transcription of either $bpsI_2$ or $bpsI_3$ could be induced during the stationary growth phase, and hence these QS systems could then assume a more prominent regulatory role.

Knowing the exact nature and proportions of the AHL signalling compounds produced by *B. pseudomallei* would

be useful in future attempts to unravel the relative contributions and cross-interactions of each of the three QS systems in regulating the virulence and social biology of this organism. Our work has highlighted a major role played by the BpsI–BpsR QS system in regulating biofilm formation in *B. pseudomallei*. However, further work needs to be done in elucidating the regulon controlled by this QS system. A comparison of transcriptome or proteome changes of a strain deficient in BpsR and the wild-type should provide valuable information and identify the quorum-regulated genes involved in the formation of biofilm by *B. pseudomallei*.

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