Growth-related changes in intracellular spermidine and its effect on efflux pump expression and quorum sensing in *Burkholderia pseudomallei*

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The *Burkholderia pseudomallei* BpeAB-OprB resistance-nodulation-division (RND) family pump effluxes aminoglycoside and macrolide antibiotics as well as acylhomoserine lactones (AHLs) involved in quorum sensing. Expression of *bpeA-lacZ* was cell density-dependent and was inducible in the presence of these compounds. Intracellular levels of spermidine and *N*-acetylspermidine increased with cell density in wild-type *B. pseudomallei* KHW, but were always lower in the *bpeAB* pump mutant at all growth phases. The significance of changes in intracellular spermidine on efflux pump expression was demonstrated by the disruption of the binding of the BpeR repressor protein to the *bpeABoprB* regulatory region *in vitro* in the presence of increasing spermidine concentrations. This was supported by dose-dependent activation of *bpeA-lacZ* transcription *in vivo* in the presence of exogenous spermidine and *N*-acetylspermidine, thus implicating the involvement of the BpeAB-OprB pump in spermidine homeostasis in *B. pseudomallei*. Consequently, inhibition of intracellular spermidine synthesis reduced the efflux of AHLs by BpeAB-OprB. Other potential therapeutic applications of spermidine synthase inhibitors include the reduction of swimming motility and biofilm formation by *B. pseudomallei*.

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INTRODUCTION

Melioidosis is a serious and potentially fatal septicaemic disease caused by the aerobic Gram-negative soil bacillus *Burkholderia pseudomallei*. The disease is endemic to south-east Asia and northern Australia, with sporadic cases occurring in other countries within latitudes 20°N and 20°S (Cheng & Currie, 2005). Therapeutic options are limited, as the pathogen is intrinsically resistant to many commonly used antibiotics, including penicillins, first and second generation cephalosporins, aminoglycosides, rifamycins and macrolides, and relapses are common (Cheng & Currie, 2005; White, 2003). Multidrug efflux pumps of the resistance-nodulation-division (RND) family, such as BpeAB-OprB, AmrAB-OprA and BpeEF-OprC, are responsible for its resistance to aminoglycosides, macrolides, trimethoprim and chloramphenicol (Chan *et al.*, 2004; Kumar *et al.*, 2006; Moore *et al.*, 1999). RND pumps also extrude diverse non-antibiotic substrates, including acylhomoserine lactones (AHLs), which are involved in quorum

sensing (Chan *et al.*, 2007; Pearson *et al.*, 1999). *B. pseudomallei* that lacks the BpeAB-OprB function is defective in quorum sensing, attenuated in virulence and also produces less biofilm (Chan & Chua, 2005).

We investigated several intermediates and products of the methionine utilization pathway involved in the synthesis of AHLs and polyamines while trying to identify other physiological substrates of the BpeAB-OprB efflux pump. Putrescine and spermidine, the predominant polyamines in Gram-negative bacteria, are known to influence gene expression either by stimulating the activity of RNA polymerase or by stabilizing ribosomal structure and modulating translational fidelity (Huang *et al.*, 1990; Igarashi & Kashiwagi, 2000; Tabor & Tabor, 1985). They affect translation by interacting directly with negatively charged DNA or RNA (Yoshida *et al.*, 1999). They also serve as free radical scavengers to protect DNA from oxidative damage (Ha *et al.*, 1998; Khan *et al.*, 1992). Polyamines protect *Escherichia coli* against hydrogen peroxide-induced oxidative stress by inducing *oxyR* and *rpoS* gene expression, and increase its survival under extremely acid conditions, a property deemed essential for successful colonization of the mammalian host (Jung & Kim, 2003a, b).

Apart from ensuring optimal growth, too many free polyamines are toxic to the bacterium because they can inhibit protein synthesis, especially during stationary phase

Abbreviations: AHL, acylhomoserine lactone; C8HSL, *N*-octanoyl-homoserine lactone; C10HSL, *N*-decanoyl-homoserine lactone; EMSA, electrophoretic mobility shift assay; 3-hydroxy-C8HSL, *N*-3-hydroxy-octanoyl-homoserine lactone; 3-hydroxy-C10HSL, *N*-3-hydroxy-decanoyl-homoserine lactone; 3-oxo-C10HSL, 3-oxo-decanoyl-homoserine lactone; 3-oxo-C14HSL, *N*-3-oxo-tetradecanoyl-homoserine lactone; RND family, resistance-nodulation-division family; RP-HPLC, reversed-phase HPLC.

and at low temperatures (Fukuchi *et al.*, 1995; Limsuwun & Jones, 2000). Hence, a balance between polyamine synthesis and catabolism is needed. Spermidine is synthesized from putrescine by spermidine synthase, which is strongly inhibited by compounds such as dicyclohexylamine and methylthiopropylamine (Pegg *et al.*, 1983). Spermidine is also acetylated by spermidine acetyltransferase to *N*-acetylspermidine, an inert and less toxic form, and bacteria that lack the acetyltransferase accumulate spermidine during the stationary phase and have reduced viability due to inhibition of protein synthesis (Seiler, 1987; Fukuchi *et al.*, 1995).

Exogenous spermidine added to culture medium increases the resistance of *Pseudomonas aeruginosa* to aminoglycosides and quinolones, although no known efflux system was found to be significantly upregulated using DNA microarray analysis (Kwon & Lu, 2006). In this study, we tracked the changes in intracellular levels of spermidine and *N*-acetylspermidine during growth of *B. pseudomallei* KHW and describe the effect of such changes on the expression of the BpeAB-OprB pump, which is involved in the efflux of mostly *N*-acetylspermidine. We also demonstrate the therapeutic potential of spermidine synthase inhibitors as antimicrobial agents that can attenuate quorum sensing, and reduce the swimming motility of and the formation of biofilms by *B. pseudomallei* KHW.

METHODS

Bacteria and plasmids. Wild-type *B. pseudomallei* KHW and its isogenic *bpeAB* deletion mutant, KHW Δ *bpeAB*, as well as the *bpeA-lacZ* reporter plasmid pCYYbpeAB, have been described previously (Chan *et al.*, 2004). *B. pseudomallei* was cultured under aerobic conditions at 37 °C in AB minimal medium supplemented with 0.2 % (w/v) glucose and 0.2 % casamino acids (Clark & Maaløe, 1967), or in Luria-Bertani (LB) medium for the motility and biofilm assays. Kanamycin and tetracycline were used at final concentrations of 200 and 25 µg ml⁻¹, respectively. The above antibiotics and the spermidine synthase inhibitors dicyclohexylamine and methylthiopropylamine were purchased from Sigma-Aldrich.

Extraction and measurement of polyamines. Wild-type *B. pseudomallei* KHW and the *bpeABoprB* mutant were cultured in AB medium containing 0.2 % (w/v) glucose alone, without casamino acids. A 10 ml volume of AB medium was inoculated with 200 µl of overnight bacterial culture and incubated at 37 °C, shaken at 100 r.p.m. After 0.5, 6, 10, 14 and 24 h, a volume of culture equivalent to 1 OD₆₀₀ unit of cells (~10⁹ cells) was removed to extract polyamines using the procedure described by Jung & Kim (2003b). One OD₆₀₀ unit of cells is equivalent to 5.6 mg wet weight of *B. pseudomallei* cells or 50 µg total protein. The cells and supernatant were separated by centrifugation at 3000 r.p.m. for 15 min. Intracellular polyamines were extracted from the cell pellet. After washing twice with 3 ml PBS, the cell pellet was resuspended in 2 ml 5 % perchloric acid (Sigma) and incubated on ice for 1 h to lyse the cells. The cell debris was removed by centrifugation at 8000 g for 10 min at 4 °C, and 1 ml 2 M NaOH and 10 µl benzoyl chloride were added to the cleared lysate for 40 min at 30 °C to derivatize the polyamines. Benzoylation caused the polyamines to absorb UV light at 254 nm and to be detected by reversed-phase HPLC (RP-HPLC) analysis. The benzoylation was stopped by adding 2 ml saturated

NaCl, and the derivatized polyamines were extracted with 2 ml diethyl ether. The organic layer (top) was collected and dried. Polyamines in the culture supernatant were similarly benzoylated and extracted using diethyl ether. The dried extracts were each solubilized in 0.1 ml methanol and filtered through a 0.45 µm pore-size filter, and 20 µl of the filtrate was applied onto a 200 mm × 4.6 mm C₁₈ column (Agilent series 1100 Hypersil ODS, particle size 5 mm). The polyamines were eluted as described by Hwang *et al.* (1997) using 50 % (v/v) methanol in water for 0.5 min, followed by a linear gradient from 50 % (v/v) to 85 % (v/v) methanol in water for 6.5 min at a flow rate of 0.8 ml min⁻¹ (Hwang *et al.*, 1997). This was followed by an isocratic profile at 85 % (v/v) for 5 min, and then a decrease over 2 min to 50 % (v/v) methanol in water at 0.8 ml min⁻¹. Peaks corresponding to the polyamines were detected using absorption spectrophotometry at a wavelength of 254 nm. Two millilitre volumes of the polyamine standards (ranging from 12.5 to 200 nM of putrescine, spermine, spermidine and *N*-acetylspermidine) (Sigma) were also benzoylated using 1 ml 2 M NaOH and 10 µl benzoyl chloride, and extracted with diethyl ether. The dried extract was also solubilized in 0.1 ml methanol, and 20 µl was injected into the HPLC column. The retention times for putrescine, spermine, spermidine and *N*-acetylspermidine were 7.3, 10.1, 9.5 and 11.1 min, respectively. The experiments were conducted in triplicate and the amounts of intracellular polyamines were expressed as pmol per 10⁹ cells (or 1 OD₆₀₀ unit), while the concentrations of polyamines in the extracellular medium were expressed in nM.

Electrophoretic mobility shift assays (EMSAs). A DNA fragment (868 bp) carrying the promoter sequences of both *bpeABoprB* and *bpeR* was amplified by PCR using the primer pair AcrABpro (5'-CTTCCTCCTCGTGCCTGTCGTA-3') and AcrA5'R (5'-GGCCACCCGCATCGTCGTA-3'). The DNA was cut using *Ava*I into two fragments, a 479 bp *bpeABoprB* promoter fragment and a 389 bp *bpeR* promoter fragment. Each fragment was recovered after gel electrophoresis in 1.2 % agarose using the GeneClean II kit (Bio 101). Recombinant BpeR repressor protein was expressed in *E. coli* M15[pREP4] harbouring the expression vector pQE30 carrying the full-length *bpeR*, and was purified under native conditions according to the manufacturer's protocol (Qiagen). EMSA was performed as described by Fried and Crothers (1981). The repressor-DNA binding reaction (10 µl) consisted of 1 µg DNA fragment, 1 µg purified BpeR, 1 µg poly-dIdC and 100 µg BSA in binding buffer (50 mM Tris-HCl, pH 8.0, 750 mM KCl, 2.5 mM EDTA, 0.5 % Triton-X, 62.5 % glycerol and 1 mM DTT). Increasing amounts of Mg²⁺ (10–1500 mM) or 0.1 nM spermidine were added where appropriate. After 30 min at room temperature, the reaction mixture was applied to a 1.2 % Tris-borate-EDTA (TBE)-agarose gel to resolve protein-DNA complexes from free DNA and visualized on a UV transilluminator after staining with ethidium bromide. Further analysis of the specificity and sensitivity of binding of BpeR to the *bpeABoprB* promoter in the presence of increasing spermidine concentrations (0.025–3.2 nM) was performed using the LightShift Chemiluminescent EMSA kit according to the manufacturer's protocol (Pierce). Briefly, 50 ng purified BpeR was mixed with 50 ng biotin-labelled DNA in binding buffer for 30 min at room temperature and then separated on a non-denaturing 5 % polyacrylamide-TBE gel. The DNA was blotted onto a nylon membrane and detected using streptavidin-horseradish peroxidase (HRP) chemiluminescence (Pierce). Non-competitor DNA, comprising the 5' end of the *bpeB* gene, was PCR-amplified using the primer pair RT-BpeBF (5'-AACGCGACGATCACGATCACA-3') and RT-BpeBR (5'-TTCAGGTGCAGAACAAAGCTGTGCG-3'). The specificity of the BpeR protein-*bpeABoprB* promoter binding in the mobility shift assay was ascertained by adding 1 µg of a 78 bp non-competitor DNA fragment to the binding reaction 10 min before the addition of labelled DNA target, and also by adding a 20-fold excess (1 µg) of unlabelled

competitor (*bpeABoprB* promoter) DNA to the binding reaction 10 min prior to adding the labelled DNA target.

Accumulation of [14 C]spermidine and [14 C]erythromycin. The level of spermidine in wild-type *B. pseudomallei* KHW, the *bpeAB* mutant (KHW Δ *bpeAB*Km) and the complemented *bpeAB* mutant (KHW Δ *bpeAB*Km/pUCP28T*bpeAB*) was measured using the method described previously (Chan *et al.*, 2004). Briefly, 16 h cultures of the bacteria in 5 ml AB medium containing 0.2% (w/v) glucose and 0.2% casamino acids were washed three times in AB medium, and 100 μ l of the washed cells was inoculated into 2 ml fresh AB medium containing 0.2% (w/v) glucose and 0.2% casamino acids. Dicyclohexylamine was added to a final concentration of 1 mM to inhibit endogenous spermidine synthesis, and [14 C]spermidine trichloride [112 mCi mmol $^{-1}$ (4.14 GBq mmol $^{-1}$) specific activity, Amersham Biosciences] was added to a final concentration of 20 nM. After incubating for 20 h at 37 $^{\circ}$ C, an aliquot of the cell culture was removed for cell density measurement at OD $_{600}$ and 500 μ l of the cell culture was harvested. The cells were washed three times in an equal volume of ice-cold 0.9% (w/v) NaCl containing 20 nM unlabelled spermidine. The cell pellet was air-dried and solubilized in 2 ml CytoScint scintillation cocktail (MP Biomedicals) and counted using an LS6500 multipurpose scintillation counter (Beckman Instruments). The amount of [14 C]spermidine retained was expressed as pmol per 10 9 cells (i.e. picomoles per the number of cells in 1 OD $_{600}$ unit) and calibrated against a standard curve of 0–100 pmol [14 C]spermidine. The experiment was performed in triplicate.

Accumulation of [14 C]erythromycin was measured using intact *B. pseudomallei* KHW cells. Briefly, 100 μ l of a 16 h culture of *B. pseudomallei* KHW was inoculated into 5 ml AB medium containing 0.2% (w/v) glucose and 0.2% casamino acids either with or without 1 mM dicyclohexylamine. [14 C]erythromycin [47 mCi mmol $^{-1}$ (1.74 GBq mmol $^{-1}$) specific activity, Perkin Elmer] was added to a final concentration of 0.1 μ g ml $^{-1}$ to the culture during early exponential phase (OD $_{600}$ ~0.5). The MIC of erythromycin for *B. pseudomallei* KHW is 128 μ g ml $^{-1}$ (Chan *et al.*, 2004). An aliquot of the culture, equivalent to 1 OD $_{600}$ unit, was removed at the start of the labelling and after 60 and 120 min. The cell pellet was solubilized in 2 ml CytoScint scintillation cocktail and the radioactivity counted as described above.

β -Galactosidase assays. The assay for β -galactosidase was performed as described by Miller (1972). Briefly, 100 μ l of an overnight culture of *B. pseudomallei* KHW harbouring pCYybpeAB was inoculated into 5 ml AB medium containing 0.2% (w/v) glucose, 0.2% casamino acids and 25 μ g tetracycline ml $^{-1}$. The culture was incubated at 37 $^{\circ}$ C for 1 h with shaking at 100 r.p.m. to OD $_{600}$ ~0.2, after which spermidine or *N*-acetylspermidine was added to give the final concentrations shown in Fig. 5. After 4 h incubation at 37 $^{\circ}$ C, the cell density (OD $_{600}$) was measured and 100 μ l of the culture was used for determining β -galactosidase activity. β -Galactosidase activity was expressed in Miller units and each assay was performed in triplicate.

Extraction and detection of radiolabelled [14 C]AHLs by RP-HPLC. AHLs produced by *B. pseudomallei* KHW were radiolabelled, extracted and analysed by RP-HPLC, as described previously (Chan *et al.*, 2007). The dried extract was dissolved in 200 μ l 20% (v/v) methanol and filtered through a 0.22 μ m pore-size membrane, and 100 μ l was injected into a C $_{18}$ reversed-phase column for HPLC analysis. Fractions of 2 ml were collected after elution at a flow rate of 1 ml min $^{-1}$ using an isocratic profile of methanol: water (50:50, v/v) for 10 min, followed by a linear gradient of 50–90% methanol in water for 25 min, and an isocratic profile of 90% methanol over 10 min. Each fraction was dried, solubilized in 2 ml CytoScint scintillation cocktail and counted using an LS6500 multipurpose scintillation counter. Peaks corresponding to *N*-

octanoyl-homoserine lactone (C8HSL), *N*-decanoyl-homoserine lactone (C10HSL), 3-oxo-decanoyl-homoserine lactone (3-oxo-C10HSL), *N*-3-oxo-tetradecanoyl-homoserine lactone (3-oxo-C14HSL), *N*-3-hydroxy-octanoyl-homoserine lactone (3-hydroxy-C8HSL), *N*-3-hydroxy-decanoyl-homoserine lactone (3-hydroxy-C10HSL) and methionine were identified using 100 μ l of the standards (1 mM), as described previously (Chan *et al.*, 2007).

Swimming motility assay. Swimming motility was tested on LB medium containing 0.3% agar and different concentrations of dicyclohexylamine (1, 5 or 10 mM) or methylthiopropylamine (1 or 5 mM). Two microlitres of a 16 h culture of *B. pseudomallei* KHW was inoculated into the centre of the agar and the plates were incubated at 30 $^{\circ}$ C for 20 h. Control plates had no dicyclohexylamine or methylthiopropylamine.

Biofilm assay. Biofilm formation was measured as the amount of bacterial cells adhering to the wells of 96-well PVC microtitre plates, using a protocol modified from O'Toole & Kolter (1998). Briefly, 100 μ l of a diluted overnight bacterial culture in LB medium (OD $_{600}$ of ~0.05) was added into each well of a 96-well PVC microtitre plate (Nunc). The wells contained different concentrations of dicyclohexylamine (1, 2, 5 and 10 mM) or methylthiopropylamine (1, 2 and 5 mM). Control wells had no dicyclohexylamine or methylthiopropylamine, while blank wells had only LB medium. After incubating for 20 h at 37 $^{\circ}$ C, planktonic cells were removed and the wells washed twice with water. Adherent cells were stained with 125 μ l 1% (w/v) crystal violet (Sigma-Aldrich) for 15 min at room temperature and then washed three times with water. The stain was dissolved in two aliquots of 100 μ l 95% (v/v) ethanol and transferred to a fresh 96-well plate to measure A $_{570}$. The assay was performed in triplicate.

RESULTS

Growth phase changes in intracellular levels of polyamines and preferential extrusion of *N*-acetylspermidine

Polyamines were extracted from ~10 9 (or 1 OD $_{600}$ unit) of *B. pseudomallei* KHW cells at different growth phases and analysed using RP-HPLC. Putrescine was the most abundant polyamine throughout the growth of *B. pseudomallei* KHW, and was at high intracellular levels (260 pmol putrescine per 10 9 cells) even during early exponential phase (6 h). This was at least eightfold higher than the corresponding levels of spermidine (13 pmol per 10 9 cells) and *N*-acetylspermidine (30 pmol per 10 9 cells). Although the amount of putrescine increased to 387 pmol per 10 9 cells in 24 h stationary phase cells, the change was not cell density-dependent (Fig. 1a). By comparison, the amounts of intracellular spermidine and *N*-acetylspermidine were very low during the lag phase but increased according to cell density during the log and stationary phases. *N*-Acetylspermidine levels also continued to increase in the bacterial cells even during the stationary phase, unlike spermidine, suggesting that spermidine was being converted to *N*-acetylspermidine during the stationary phase (Fig. 1a). In spite of its abundance intracellularly, putrescine was not extruded into the culture medium. Only spermidine and *N*-acetylspermidine were extruded by *B. pseudomallei*, and *N*-acetylspermidine was the predom-

Fig. 1. (a) Growth phase changes in intracellular polyamine content in wild-type *B. pseudomallei* KHW cultured in AB minimal medium containing 0.2% glucose. The polyamines were extracted from the cell pellet of $\sim 10^9$ (1 OD_{600} unit) *B. pseudomallei* KHW cells. (b) Growth phase changes in the extracellular concentrations of polyamines in wild-type *B. pseudomallei* KHW. The polyamines were extracted from 1 ml bacterial culture supernatant. Concentrations of extracellular polyamines were expressed as pmol (ml culture medium) $^{-1}$ (or nM). Δ , Cell density (OD_{600}); $\times$, putrescine; $\blacksquare$, N-acetylspermidine; $\square$, spermidine. Values shown are the mean from three independent experiments; error bars, SD.

inant polyamine extruded (Fig. 1b). Extracellular concentrations of N-acetylspermidine were 15-fold higher than those of spermidine in 24 h cultures, although the changes in extracellular concentrations of both polyamines were clearly cell density-dependent.

Involvement of the BpeAB-OprB pump in extrusion of N-acetylspermidine and spermidine

Evidence that the BpeAB-OprB pump is involved in the extrusion of polyamines was supported by data showing a fivefold reduction in extracellular N-acetylspermidine concentration (123 nM) as well as undetectable levels of spermidine in the culture medium of the stationary phase (24 h) *bpeAB* mutant as compared with the wild-type (Fig. 2b). When exposed to radiolabelled [^{14}C]spermidine, we also

detected threefold higher retention of exogenous [^{14}C]spermidine in the *bpeAB* mutant after 20 h when compared with wild-type cells (Fig. 3). The level of exogenous [^{14}C]spermidine in the complemented *bpeAB* mutant expressing *bpeAB* from plasmid pUCP28T*bpeAB* was reduced to almost wild-type levels (Fig. 3). We do not have data on the retention of exogenous radiolabelled N-acetylspermidine, as the compound was not commercially available. It is also interesting to note the reduced levels of spermidine and N-acetylspermidine in the *bpeAB* mutant as compared with the wild-type (Figs 1a and 2a). The intracellular levels of spermidine and N-acetylspermidine in the *bpeAB* mutant at 24 h were three- to fourfold lower than the levels in the wild-type, although the intracellular levels of putrescine were unaffected. Although we attributed the reduction in the total amount of extracellular spermidine and N-acetylspermidine

Fig. 2. (a) Changes in intracellular polyamine concentrations in the *bpeAB* mutant during growth. Polyamines were extracted from the cell pellet of $\sim 10^9$ ($1 OD_{600}$ unit) *B. pseudomallei* KHW $\Delta bpeAB$ cells cultured in AB minimal medium containing 0.2% glucose. (b) Growth phase changes in extracellular concentrations of polyamines in the *bpeAB* pump mutant. Polyamines were extracted from 1 ml bacterial culture supernatant. The amounts of extracellular polyamines were expressed as nmol (l culture medium) $^{-1}$. Δ , Cell density (OD_{600}); $\times$, putrescine; $\blacksquare$, N-acetylspermidine; $\square$, spermidine. Values shown are the mean from three independent experiments; error bars, SD.

in the *bpeAB* mutant to an impaired efflux, the reduced intracellular levels of spermidine and N-acetylspermidine in the *bpeAB* mutant suggest that an impaired BpeAB-OprB function also has a direct effect on spermidine biosynthesis. This effect seemed to be specific to the spermidine biosynthesis pathway, as the intracellular levels of putrescine were largely unaffected in the *bpeAB* mutant.

High levels of spermidine disrupt the binding of protein to DNA *in vitro*

To determine the significance of changes in intracellular spermidine concentrations on gene expression, we used EMSA to show that increasing intracellular spermidine concentrations could disrupt the binding of regulatory

proteins to DNA *in vitro*. Binding of the BpeR repressor protein to both the *bpeABoprB* promoter and the *bpeR* promoter was observed in the absence of spermidine but, in both instances, the binding was disrupted in the presence of 0.1 nM spermidine (Fig. 4a, b). It was further verified using different concentrations of spermidine that the binding of the BpeR repressor to the *bpeABoprB* promoter occurred only if the spermidine concentration was 0.1 nM or less, but not when the concentration was above 0.1 nM (Fig. 4c). The EMSA experiment using a biotinylated DNA probe and chemiluminescent detection was significantly more sensitive than the method using ethidium bromide to detect shifted bands by agarose gel electrophoresis in Fig. 4(a), and could hence detect an additional higher-mobility shifted band in lanes 1 and 2 in Fig. 4(c). However, the lower shifted band

Fig. 3. Accumulation of exogenous [¹⁴C]spermidine in wild-type *B. pseudomallei* KHW cells, the *bpeAB* mutant and the complemented *bpeAB* mutant. The amounts of [¹⁴C]spermidine in wild-type *B. pseudomallei* KHW, the KHWΔ*bpeAB*Km mutant and the complemented KHWΔ*bpeAB*Km mutant were measured at 20 h after adding 20 nM [¹⁴C]spermidine and 1 mM dicyclohexylamine to the culture medium. Values shown are the mean from three independent experiments; error bars, SD.

appeared to be non-specific, and was possibly due to the presence of non-specific contaminating His-tagged proteins or truncated BpeR protein in the protein preparation, since the upper band was the predominant shifted product in the presence of 0.1 nM spermidine (lane 3). We also challenged the binding using excess non-competitor *bpeB* DNA to ascertain specificity of the binding assay. BpeR did not bind to the non-competitor *bpeB* DNA even in the absence of spermidine. Furthermore, excess unlabelled competitor *bpeABoprB* promoter DNA competed with the binding of BpeR to the labelled *bpeABoprB* promoter DNA in the absence of spermidine (Fig. 4c). Binding of BpeR repressor to the *bpeABoprB* promoter could also be disrupted by gradually increasing the concentration of Mg²⁺ in the binding buffer from 10 to 100 mM, but the Mg²⁺ concentration needed to disrupt the protein–DNA interaction was at least 10⁵-fold higher than using spermidine (data not shown). We conclude that only small changes in intracellular spermidine concentration are sufficient to affect protein–DNA interaction.

Exogenous spermidine and *N*-acetylspermidine induce *bpeA–lacZ* expression *in vivo* in a dose-dependent manner

Next, we showed that transcription of *bpeA–lacZ* *in vivo* could be induced by spermidine. Transcription of *bpeA–lacZ* was induced in a dose-dependent manner by the addition of exogenous spermidine to wild-type *B. pseudo-*

mallei KHW harbouring plasmid pCYY*bpeAB* (Fig. 5a). Similar dose-dependent induction of *bpeA–lacZ* transcription was observed in the presence of exogenous *N*-acetylspermidine (Fig. 5b). A comparison of the induction levels of *bpeA–lacZ* transcription in the presence of spermidine and *N*-acetylspermidine revealed that expression of *bpeA–lacZ* was more sensitive to changes in concentrations of *N*-acetylspermidine, requiring <1 nM exogenous *N*-acetylspermidine to achieve maximum induction as compared with 5 nM exogenous spermidine (Fig. 5). Interestingly, no induction in the transcription of *bpeA–lacZ* was observed in the presence of exogenous putrescine, even up to 50 nM (data not shown).

Inhibition of spermidine synthesis reduces efflux of [¹⁴C]erythromycin and AHLs

Since spermidine could induce expression of *bpeABoprB*, we also explored the possibility of using spermidine synthase inhibitors to reduce the efflux of substrates by the BpeAB–OprB pump. Efflux of [¹⁴C]erythromycin was reduced by at least 4.5-fold in *B. pseudomallei* KHW cultured in the presence of 1 mM dicyclohexylamine and a subinhibitory concentration (0.1 μg ml^{−1}) of [¹⁴C]erythromycin when compared with bacteria cultured in medium without dicyclohexylamine (Fig. 6). As the BpeAB–OprB pump is involved in the efflux of AHLs, dicyclohexylamine could also similarly reduce the efflux of AHLs and thus attenuate quorum sensing in *B. pseudomallei* KHW (Chan & Chua, 2005). Analysis of ¹⁴C-labelled AHLs extracted from the culture supernatant of 24 h *B. pseudomallei* KHW cultures revealed undetectable levels of all six AHLs, C8HSL, C10HSL, 3-hydroxy-C8HSL, 3-oxo-C10HSL, 3-hydroxy-C10HSL and 3-oxo-C14HSL, when *B. pseudomallei* KHW was cultured in the presence of 1 mM dicyclohexylamine (Fig. 7). Hence, spermidine synthase inhibitors can attenuate quorum sensing in *B. pseudomallei* KHW.

Inhibition of spermidine synthesis reduces swimming motility and biofilm formation

We also showed that intracellular spermidine levels can also affect swimming motility and biofilm formation in *B. pseudomallei* KHW. Swimming motility was progressively reduced in *B. pseudomallei* KHW cultured in LB medium containing 1, 5 and 10 mM dicyclohexylamine as well as 5 mM methylthiopropylamine (Fig. 8a). Biofilm formation, a process which is dependent on bacterial motility, was also significantly reduced in *B. pseudomallei* KHW cultured in LB medium containing 1, 2, 5 and 10 mM dicyclohexylamine, as well as in LB medium containing 1, 2 and 5 mM methylthiopropylamine (Fig. 8b).

DISCUSSION

Stationary phase *B. pseudomallei* KHW produced higher levels of *N*-acetylspermidine than spermidine, although

Fig. 4. EMSAs showing the effects of spermidine and Mg^{2+} concentrations on protein–DNA binding *in vitro*. *In vitro* binding of the BpeR protein to the *bpeABoprB* promoter (a) and the *bpeR* promoter (b) was disrupted in the presence of 0.1 nM spermidine. (c) Titration of the *in vitro* binding of the BpeR protein to the *bpeABoprB* promoter with increasing concentrations of spermidine ranging from 0.025 to 3.2 nM. Lanes: 1–3, binding of BpeR to the labelled *bpeABoprB* promoter at spermidine concentrations up to 0.1 nM; 4–8, disruption of the BpeR–*bpeAB* promoter binding at spermidine concentrations above 0.1 nM; 9, disruption of the binding of BpeR protein to the labelled *bpeABoprB* promoter by excess unlabelled competitor *bpeABoprB* promoter; 10, unbound labelled *bpeABoprB* promoter alone; 11, no competition in the binding of BpeR to the labelled *bpeABoprB* promoter in the presence of excess unlabelled non-competitor *bpeB* DNA; 12, binding of BpeR to the labelled *bpeABoprB* promoter in the absence of spermidine.

the levels of both increased significantly during the growth of the bacteria. Conversion of spermidine to *N*-acetylspermidine seems to be a response to stress, such as limiting nutrients or the oxidative stress associated with entry to stationary phase, as observed in *E. coli* cells (Carper *et al.*, 1991). Unlike spermidine, *N*-acetylspermidine is physiologically inert and does not bind to RNA or stimulate *in vitro* translation. Therefore, active conversion of spermidine to *N*-acetylspermidine by spermidine acetyltransferase would be an effective mechanism to reduce the toxicity associated with high levels of spermidine (Kakegawa *et al.*, 1991). Although speculative, it is possible that cell density-dependent changes in intracellular spermidine levels, which are linked to methionine utilization and the production of *S*-adenosylmethionine (SAM), function as a metabolic sensor for *B. pseudomallei* to regulate gene expression and protein

synthesis in a growth phase-dependent manner. Spermine, which is present in eukaryotic cells, has not been detected in *B. pseudomallei* KHW and other bacteria (Igarashi & Kashiwagi, 2000).

In *B. pseudomallei* KHW, acetylation seems to serve not only to detoxify spermidine but also to facilitate its extrusion from the bacterium. An *E. coli* *speG* mutant lacking spermidine acetyltransferase shows growth inhibition during cold shock due to the accumulation of high levels of spermidine. The growth inhibition is attributed to ribosome inactivation caused by a replacement of ribosome-bound Mg^{2+} by spermidine (Limsuwun & Jones, 2000). The *E. coli* *speG* mutant is also sensitive to exogenous spermidine and exhibits decreased cell viability, especially when exogenous spermidine is added to 24 h stationary phase cells (Fukuchi *et al.*, 1995). It is possible

Fig. 5. Induction of *bpeA-lacZ* expression in *B. pseudomallei* KHW by exogenous spermidine and *N*-acetylspermidine. (a) Dose-dependent induction of *bpeA-lacZ* expression in the presence of increasing concentrations of exogenous spermidine ranging from 0.05 to 10 nM (○). (b) Dose-dependent induction of *bpeA-lacZ* expression in the presence of increasing concentrations of exogenous *N*-acetylspermidine ranging from 0.01 to 1 nM (□). Values shown are the mean from three independent experiments; error bars, SD.

that spermidine in *B. pseudomallei* is also actively acetylated to overcome the inhibition of protein synthesis that might otherwise occur during stationary phase. It has also been noted that bacteria such as *E. coli* do not have any spermidine deacetylating activity or polyamine oxidase activity, so *N*-acetylspermidine cannot be recycled but has to be extruded from the cell. Our data support the view that spermidine is actively acetylated and extruded from *B. pseudomallei* as *N*-acetylspermidine by the BpeAB-OprB pump, although some of this inert polyamine remained in the cells during stationary phase. A plausible explanation for the preferential efflux of acetylated spermidine by the BpeAB-OprB pump would be a change in the structural properties of spermidine at physiological pH that reduces its binding affinity to the pump when compared with *N*-acetylspermidine (Karahalios *et al.*, 1998). We also noted the presence of low levels of *N*-acetylspermidine (~20 % of wild-type levels) in the extracellular medium of the *bpeAB* mutant and attribute

this to an involvement of a secondary mechanism other than BpeAB-OprB in its efflux.

Together with Mg^{2+} , polyamines account for the majority of the intracellular cationic charges, and they are essential for normal cell growth and viability (Canellakis *et al.*, 1979). Although the effect of polyamines on cell growth is believed to occur at the level of translation, we have shown that polyamines can also regulate gene expression at the transcriptional level *in vitro* by affecting the binding of regulatory proteins to DNA. The polyamine modulon in *E. coli* has been defined as a group of genes whose expression is regulated by polyamines and comprises several transcription factors whose translation is affected by polyamines (Igarashi & Kashiwagi, 2006). We have shown that small changes in spermidine concentration affect the binding of the BpeR repressor protein to the operator regions of the *bpeABoprB* and *bpeR* promoters *in vitro*. The ability of spermidine to induce *bpeA-lacZ* transcription *in*

Fig. 6. Dicyclohexylamine increases the retention of [^{14}C]erythromycin in *B. pseudomallei* KHW cells. ■, *B. pseudomallei* KHW cells cultured in AB medium containing 0.2% (w/v) glucose and 1 mM exogenous dicyclohexylamine; ○, *B. pseudomallei* KHW cells cultured in AB medium containing 0.2% (w/v) glucose alone. Values shown are the mean from three independent experiments; error bars, SD.

in vivo in a dose-dependent manner supports the *in vitro* data. It is also possible that changes in intracellular spermidine affect expression of many other genes.

We propose that spermidine, which is polycationic, interacts with the binding of BpeR to DNA in the same manner that Mg^{2+} affects protein–DNA interaction, albeit with much higher sensitivity. Therefore, depending on the metabolic state of the bacteria, subtle changes in intracellular spermidine concentrations may be sufficient to mediate

changes in gene expression in *B. pseudomallei* KHW. Using dicyclohexylamine to inhibit intracellular spermidine synthesis, we showed that efflux of [^{14}C]erythromycin by the BpeAB-OprB pump could be reduced significantly, thus uncovering a mechanism for modulating efflux pump activity. Real-time PCR transcriptional analysis of eight putative RND efflux pumps in stationary phase *B. pseudomallei* KHW cultured in AB medium containing dicyclohexylamine revealed significant downregulation in the transcription of all the pumps, including BpeAB-OprB, AmrAB-OprB and BpeEF-OprC (data not shown).

Due to the involvement of BpeAB-OprB in the efflux of AHLs, we showed that inhibiting intracellular spermidine synthesis can also block the extracellular production of AHLs required for quorum sensing in *B. pseudomallei*. Optimal intracellular spermidine concentrations are also required for cellular functions of *B. pseudomallei* KHW other than cell growth. Inhibition of intracellular spermidine synthesis also reduced biofilm formation and swimming motility in *B. pseudomallei* KHW. It is possible that some of these phenotypes can be attributed to a direct effect of spermidine on the BpeAB-OprB pump, which has a significant impact on antibiotic resistance, quorum sensing, biofilm formation and virulence when impaired (Chan & Chua, 2005). Dicyclohexylamine also inhibits the synthesis of spermidine and growth in *P. aeruginosa*, accompanied by inhibition of motility and synthesis of flagella, while increasing concentrations of norspermidine in *Vibrio cholera* activate biofilm formation (Karatan *et al.*, 2005; Paulin *et al.*, 1986). Considering the importance of quorum sensing in *B. pseudomallei* virulence and biofilm formation, spermidine synthase inhibitors could be novel anti-infective and anti-biofilm agents against *B. pseudomallei* (Chan & Chua, 2005).

Fig. 7. Dicyclohexylamine attenuates quorum sensing by inhibiting production of extracellular AHLs. Analysis of extracellular AHLs extracted from stationary phase (24 h) *B. pseudomallei* KHW cultured in AB medium only (●) and in AB medium containing 1 mM dicyclohexylamine (□). Peak positions 1, 2, 3, 4, 5, 6 and 7 correspond to the elution profiles of methionine, C8HSL, C10HSL, 3-hydroxy-C8HSL, 3-oxo-C10HSL, 3-hydroxy-C10HSL and 3-oxo-C14HSL, respectively. Values shown are the mean from three independent experiments; error bars, SD.

Fig. 8. Effect of spermidine on *B. pseudomallei* and biofilm formation. (a) Swimming motility of *B. pseudomallei* KHW was reduced in LB medium containing 0.3% agar and increasing concentrations of spermidine synthase inhibitors. (i) LB medium only; (ii–iv) with 1, 5 and 10 mM dicyclohexylamine; (v, vi) with 1 and 5 mM methylthiopropylamine. (b) *B. pseudomallei* KHW formed less biofilm in LB medium containing increasing concentrations of spermidine synthase inhibitors. Black and grey bars show amounts of biofilm formed in the presence of dicyclohexylamine and methylthiopropylamine, respectively. Values shown are the mean from four independent experiments; error bars, SD.

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