Control of Quorum Sensing by a *Burkholderia pseudomallei* Multidrug Efflux Pump[▽]

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The *Burkholderia pseudomallei* KHW quorum-sensing systems produced *N*-octanoyl-homoserine lactone, *N*-decanoyl-homoserine lactone, *N*-(3-hydroxy)-octanoyl-homoserine lactone, *N*-(3-hydroxy)-decanoyl-homoserine lactone, *N*-(3-oxo)-decanoyl-homoserine lactone, and *N*-(3-oxo)-tetradecanoyl-homoserine lactone. The extracellular secretion of these acyl-homoserine lactones is dependent absolutely on the function of the *B. pseudomallei* BpeAB-OprB efflux pump.

Burkholderia pseudomallei, a gram-negative soil bacillus, is the causative agent of melioidosis, a severe and potentially fatal emerging tropical infectious disease. Its intrinsic resistance to many antibiotics is attributed mostly to multiple multidrug efflux pumps, such as AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC, which efflux aminoglycosides, macrolides, chloramphenicol, and trimethoprim (4, 12, 14). We hypothesized that these pumps also efflux physiological compounds that could compromise the fitness of the bacterium when accumulated to high intracellular concentrations and propose that this exerts a positive selection pressure for persistence even in the absence of antimicrobials (11).

B. pseudomallei is reported to produce up to six different types of acyl-homoserine lactones (acyl-HSLs), the composition of which may differ slightly from strain to strain. For instance, strain PP844 secreted *N*-octanoyl-homoserine lactone (C8HSL), *N*-(3-hydroxy)-octanoyl-homoserine lactone (3-hydroxy-C8HSL), *N*-(3-oxo)-octanoyl-homoserine lactone (3-oxo-C8HSL), *N*-decanoyl-homoserine lactone (C10HSL), *N*-(3-hydroxy)-decanoyl-homoserine lactone (3-hydroxy-C10HSL), and *N*-(3-hydroxy)-dodecanoyl-homoserine lactone (3-hydroxy-C12HSL), while strain DD503, an *amrAB-oprA* efflux pump operon deletion mutant derived from 1026b, secreted only C8HSL, 3-hydroxy-C8HSL, and C10HSL (13, 17–19). It is unclear how acyl-HSLs move across the *B. pseudomallei* cell membranes and into the extracellular compartment, although in *Pseudomonas aeruginosa*, the shorter-chain acyl-HSLs appear to do so by diffusion while the longer-chain acyl-HSLs are secreted by multidrug efflux pumps (1, 15). In *B. pseudomallei* KHW, the expression of *bpeAB-lacZ* was induced by acyl-HSLs, and the *bpeAB* mutant failed to secrete any

extracellular acyl-HSLs when it was cross-streaked against the JB525 reporter strain (3).

Extracellular secretion of acyl-HSLs is dependent on BpeAB-OprB. In order to ascertain whether a blockage in the efflux mechanism, an inhibition of acyl-HSL synthesis, or both had contributed to the absence of extracellular acyl-HSLs in the *bpeAB* mutant, we compared the acyl-HSLs produced in cell supernatants of *B. pseudomallei* before and after the bacterial cells were permeabilized by a freeze-thaw procedure. Wild-type *B. pseudomallei* KHW, KHW carrying a plasmid overexpressing the BpeR repressor, the *bpeAB* null mutant, and a complemented *bpeAB* mutant were used in the comparison (Table 1), and a modified cross-streak bioassay was used for detection of acyl-HSLs (2). The culture supernatants of unpermeabilized wild-type KHW and the complemented *bpeAB* mutant contained acyl-HSLs but not those of the *bpeAB* mutant and the *bpeR*-overexpressing strain, both of which had impaired BpeAB-OprB function (Fig. 1, lanes U). Acyl-HSLs were detected in the cell supernatants of the *bpeAB* mutant and the *bpeR*-overexpressing strain only after the bacterial cells were permeabilized by five cycles of freeze-thaw (−80°C for 3 min and 37°C for 1 min). This demonstrates that the *bpeAB* mutant and the *bpeR*-overexpressing strain were defective in the extracellular secretion, but not intracellular synthesis, of acyl-HSLs (Fig. 1, lanes P). It was not possible to ascertain from the experiment whether the synthesis of a specific acyl-HSL(s) might be affected, and the bioassay was also not informative about the relative abundance of acyl-HSLs in the wild-type and *bpeAB* mutant cells.

BpeAB-OprB is required for efflux but not for influx of acyl-HSLs during quorum sensing. The consensus is that longer-chain acyl-HSL autoinducers produced by gram-negative bacteria, such as *N*-(3-oxo)-dodecanoyl-homoserine lactone (3-oxo-C12HSL) in *P. aeruginosa*, probably require active export, while short-chain acyl-HSLs, such as *N*-butanoyl-homoserine lactone (C4HSL) diffuse freely across the cell membrane (15). Early-log-phase cultures of wild-type *B.*

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TABLE 1. Bacterial strains used in this study

Strain	Characteristics ^a	Source or reference
<i>E. coli</i> strains ^b		
DH5 α	<i>endA hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 deoR</i> [ϕ 80dlac Δ (lacZ)M15]	Gibco-BRL
JB525	Indicator bacterium used for detecting acyl-HSLs; derivative of <i>E. coli</i> MC1000 harboring plasmid pJBA132 Tet ^r	2
<i>B. pseudomallei</i> strains		
KHW	Wild-type parental strain; clinical isolate	5
KHW Δ <i>bpeAB</i>	<i>bpeAB</i> deletion mutant derived from KHW; Kan ^r	4
KHW Δ <i>bpeAB</i> (pUCP28 <i>TbpeAB</i>)	<i>bpeAB</i> deletion mutant harboring pUCP28T carrying full-length <i>bpeAB</i> ; Kan ^r Tmp ^r	4
KHW <i>bpeR::Km</i>	<i>bpeR</i> null mutant derived from KHW by insertion of a kanamycin resistance cassette into <i>bpeR</i> ; Kan ^r	4
KHW(pUCP28 <i>TbpeR</i>)	Wild-type strain overexpressing <i>bpeR</i> ; KHW harboring pUCP28T carrying full-length <i>bpeR</i> ; Tmp ^r	3

^a Kan, kanamycin; Tmp, trimethoprim; Tet, tetracycline; Amp, ampicillin.

^b *E. coli* strains were cultured in LB broth and maintained on LB agar (Becton Dickinson, Cockeysville, MD).

pseudomallei KHW and the *bpeAB* mutant were exposed to exogenous [¹⁴C]acyl-HSLs, and the intracellular levels of [¹⁴C]acyl-HSLs were measured at 30, 60, and 90 min thereafter. After 60 min, the amounts of intracellular [¹⁴C]acyl-HSLs were ~2.5-fold higher for the *bpeAB* null mutant and wild-type cells that carried a plasmid overexpressing the BpeR repressor than for the wild type (Fig. 2). This observation was similar to the accumulation [¹⁴C]erythromycin in the *bpeAB* mutant, which we reported previously (4). The *bpeR* null mutant that overexpressed *bpeAB* also accumulated less [¹⁴C]acyl-HSLs than did the *bpeAB* mutant but to levels similar to those of the wild-type (Fig. 2). We attribute the similar levels of accumulation of [¹⁴C]acyl-HSLs in the wild type and *bpeR* mutant to the possibility that *bpeAB* gene expression may not be linearly correlated with BpeAB-OprB pump activity (Fig. 2). Previous data comparing the susceptibilities of wild-type KHW and the *bpeR* null mutant to antimicrobial agents had shown an increase of 16-fold in the MIC of gentamicin but of only 2-fold in the MIC of streptomycin in the *bpeR* null mutant compared to

the wild type, although both antibiotics were substrates of BpeAB-OprB (3). It is also possible that, after 60 min of incubation, some of the accumulated ¹⁴C-labeled product in the *bpeAB* mutant and the *bpeR*-overexpressing strain might have degraded, and the radiolabel detected could have included metabolites of acyl-HSLs that were also dependent on BpeAB-OprB efflux. BpeAB-OprB seemed to be required specifically for the efflux of acyl-HSLs but had no effect on the reentry of acyl-HSLs into the bacterial cell during quorum sensing, during which they are most likely to enter the cells by passive diffusion.

FIG. 1. Detection of extracellular and intracellular acyl-HSLs produced by *B. pseudomallei* with the *E. coli* JB525 bioassay. Overnight (16-h) cultures of *B. pseudomallei* in 5 ml of AB medium (6) containing 0.2% glucose were centrifuged at 3,000 rpm for 15 min. Secreted acyl-HSLs were detected by adding 50 μ l of the cell supernatant to the wells labeled U on LB agar. The cell pellets, each resuspended in 1 ml of fresh AB medium, were freeze-thawed (five cycles at -80°C for 3 min and 37°C for 1 min) to permeabilize the cells, and 50 μ l of the cell extract was then added to the adjacent wells, labeled P. A gradient of acyl-HSLs diffusing from the wells after incubation at 30°C for 20 h was detected as green fluorescence by a row of 2- μ l spots of *E. coli* JB525 when illuminated under UV. The direction of diffusion from the well is indicated by the arrow. *E. coli* DH5 α was included as a negative control.

FIG. 2. Intracellular accumulation of exogenous [¹⁴C]acyl-HSLs. Radiolabeled [¹⁴C]acyl-HSLs were prepared by adding 5 μ Ci of L-[1-¹⁴C]methionine (Sigma) to a 16-h culture of *B. pseudomallei* in AB medium containing 20 mM glycerol for 30 min. [¹⁴C]acyl-HSLs were then extracted from the culture supernatants by acidified ethyl acetate, according to a procedure described previously (16). Two hundred microliters of the [¹⁴C]acyl-HSLs was added to early-log-phase cells (optical density at 600 nm, ~0.2), and 0.2-ml aliquots were removed after 30, 60, and 90 min for scintillation counting. The cells were washed three times with saline containing 100 nM C8HSL to remove extracellular radiolabeled acyl-HSLs, air dried, and solubilized in 2 ml of scintillation cocktail (Amersham Biosciences) for liquid scintillation counting with an LS6500 multipurpose scintillation counter (Beckman Instruments Inc., Fullerton, CA). White bars, wild-type *B. pseudomallei* KHW; black bars, KHW Δ *bpeAB* mutant; stippled bars, KHW *bpeR::Km* null mutant; gray bars, KHW(pUCP28*TbpeR*).

FIG. 3. Comparison of intracellular and extracellular acyl-HSLs produced by wild-type *B. pseudomallei* KHW and the *bpeAB* null mutant by reversed-phase HPLC. Radiolabeled [^{14}C]acyl-HSLs was extracted from 5-ml cultures of KHW, KHW $\Delta bpeAB$ mutant, and KHW(pUCP28*bpeR*), as described in the legend to Fig. 2. The extract was dried and dissolved in 200 μl of 20% (vol/vol) methanol before the application of 100 μl into a C_{18} reversed-phase HPLC column. Two-milliliter fractions were collected during elution at a flow rate of 1 ml/min, with an isocratic profile of methanol-water (50:50, vol/vol) for 10 min, followed by a linear gradient of 50 to 90% methanol in water for 25 min and an isocratic profile of 90% methanol over 10 min. (A) [^{14}C]acyl-HSLs secreted into the culture supernatants of untreated stationary-phase bacterial cultures. (B) [^{14}C]acyl-HSLs extracted from freeze-thaw-permeabilized bacterial cells. Peaks 1, 2, 3, 4, 5, 6, and 7 were detected with a Radiomatic 500TR Radioisotope HPLC detector (Perkin-Elmer) and correspond to the elution profiles of L-methionine, C8HSL, C10HSL, 3-hydroxy-C8HSL, 3-oxo-C10HSL, 3-hydroxy-C10HSL, and 3-oxo-C14HSL, respectively. The HPLC profiles of acyl-HSLs extracted from culture supernatants of wild-type KHW and its *bpeAB* deletion mutant are represented by closed and open squares, respectively. C8HSL, C10HSL, and methionine were purchased from Sigma, while 3-oxo-C10HSL and 3-oxo-C14HSL were synthesized by a microwave-assisted solid-phase synthetic route to acyl-homoserine lactones (8) and 3-hydroxy-C8HSL and 3-hydroxy-C10HSL were synthesized by the Reformatsky reaction (9).

Involvement of BpeAB-OprB in the efflux of acyl-HSLs. Using reversed-phase high-performance liquid chromatography (HPLC) to profile acyl-HSLs extracted from the culture supernatants of the wild type and the *bpeAB* null mutant with acidified ethyl acetate, it was possible to ascertain which acyl-HSL(s) was effluxed by BpeAB-OprB. Seven major peaks were detected in both the cell culture supernatants and freeze-thawed extracts of the wild-type cells (Fig. 3A and B, respectively). Peaks 1, 2, 3, 4, 5, 6, and 7 were identified as L-methionine, C8HSL, C10HSL, 3-hydroxy-C8HSL, *N*-(3-oxo)-decanoyl-homoserine lactone (3-oxo-C10HSL), 3-hydroxy-C10HSL, and *N*-(3-oxo)-tetradecanoyl-homoserine lactone (3-oxo-C14HSL), respectively, after comparisons of retention times with those of synthetic acyl-HSL standards and methionine. The compounds eluted in peaks 2 to 7 were also confirmed as acyl-HSLs by applying 50 μl of each fraction in the

modified cross-streak bioassay described earlier (data not shown). Strain KHW differed from strain DD503 in the secretion of 3-oxo-C10HSL, 3-hydroxy-C10HSL, and 3-oxo-C14HSL, in addition to the three acyl-HSLs also secreted by DD503 (18). Among these, 3-oxo-C10HSL was unique to KHW. As the AmrAB-OprA efflux pump was defective in DD503, the extracellular secretion of 3-oxo-C10HSL by KHW would suggest that it might be effluxed by AmrAB-OprA, which coincidentally shares similar antimicrobial substrates with BpeAB-OprB (4, 14). Another *B. pseudomallei* strain, PP844, was also reported to secrete six acyl-HSLs when cultured in tryptic soy broth, although it differed from KHW in its production of 3-oxo-C8HSL and 3-hydroxy-C12HSL, both of which were not detected in KHW and DD503 (13). Although this conclusion is speculative, it is possible that the synthesis of acyl-HSLs, which are derived from methionine and acyl-chain

FIG. 4. Comparison of reversed-phase HPLC profiles of intracellular and extracellular acyl-HSLs produced by the *B. pseudomallei* *bpeR* null mutant and wild-type KHW carrying a plasmid overexpressing the BpeR repressor. (A) [^{14}C]acyl-HSLs extracted from supernatants of untreated stationary-phase bacterial cultures. (B) [^{14}C]acyl-HSLs extracted from bacterial cells permeabilized by repeated freeze-thawing in fresh AB medium. 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. Both the *bpeR* null mutant and the wild-type KHW carrying a plasmid overexpressing the BpeR repressor failed to synthesize any 3-oxo-C14HSL (peak 7). The HPLC profiles of acyl-HSLs extracted from culture supernatants of KHW *bpeR* and KHW(pUCP28*bpeR*) are represented by black circles and gray circles, respectively.

donors, may be affected by culture conditions, a possibility that should be investigated.

In contrast to wild-type *B. pseudomallei* KHW, none of the acyl-HSL species was detected in the culture supernatant of the *bpeAB* mutant, which corroborates the data from the cross-streak bioassays (Fig. 3A). Only C8HSL—none of the other five acyl-HSLs—was detected in the cell extracts of the permeabilized *bpeAB* mutant (Fig. 3B). The intracellular level of C8HSL, which was reduced in the *bpeAB* mutant compared to the wild type, may be attributed to a negative impact on the synthesis of C8HSL by BpsI as a result of the lack of quorum sensing in the *bpeAB* mutant. This is supported by our previous data, which also showed a significant reduction in the expression of *bpsI-lacZ* in the *bpeAB* mutant compared to the wild type (3). As the *bpeAB* mutant synthesized only C8HSL, this would suggest that the BpsIR (PmlIR) quorum-sensing system probably controls the expression of the other *bpmIR* systems, which produce C10HSL, 3-hydroxy-C8HSL, 3-oxo-C10HSL, 3-hydroxy-C10HSL, and 3-oxo-C14HSL. It might also be inferred that inhibition of the BpeAB-OprB efflux pump would be beneficial, because it enhances the antimicrobial properties of aminoglycoside and macrolide antibiotics and attenuates *B. pseudomallei* virulence by blocking quorum sensing. BpeAB-

OprB is expressed in 24 of the 26 *B. pseudomallei* isolates that we have tested (data not shown).

Effect of BpeR on the efflux of acyl-HSLs. The *bpeR* null mutant, which overexpressed *bpeAB*, differed from the wild type in that it secreted only C8HSL, C10HSL, and 3-hydroxy-C8HSL (Fig. 4A). The absence of intracellular 3-hydroxy-C10HSL, 3-oxo-C10HSL, and 3-oxo-C14HSL in the *bpeR* mutant would suggest that an optimal level of BpeR repressor, probably via its regulation of *bpeAB* expression, was necessary to control the synthesis of these acyl-HSLs. The exact mechanism by which BpeR regulates expression of *bpeAB-oprB*, and whether it might also regulate the expression of another resistance-nodulation-division pump, such as AmrAB-OprA, which we had earlier alluded to in the secretion of 3-oxo-C10HSL, is still unclear. Basal expression of *bpeAB* was increased in the *bpeR* null mutant but could still increase further in the presence of erythromycin, a BpeAB-OprB substrate. It is not clear how the BpeR-dependent and BpeR-independent mechanisms interact with each other (3).

Wild-type KHW carrying a plasmid that overexpresses the BpeR repressor showed significantly reduced *bpeAB* transcription and enhanced susceptibility to gentamicin, streptomycin, and erythromycin, thus suggesting a reduced BpeAB-OprB

efflux function in these cells (3). Therefore, the detection of 3-oxo-C10HSL and 3-hydroxy-C10HSL in the supernatants of these stationary-phase cultures was intriguing (Fig. 4A). From the observation that *B. pseudomallei* DD503 secreted 3-hydroxy-C10HSL only when *pmlI*, *bpmI*₂, or *bpmI*₃ was overexpressed and 3-oxo-C14HSL only when *bpmI*₂ or *bpmI*₃ was overexpressed, it is plausible that the BpeR repressor might have had an indirect influence on the expression of the auto-inducer synthases responsible for syntheses of these two acyl-HSLs. As *B. pseudomallei* KHW containing a plasmid that overexpressed the BpeR repressor was highly attenuated in cytotoxicity and cell invasion, produced less siderophores and phospholipase C, and was impaired in biofilm formation compared to the wild type, it may be inferred that 3-oxo-C10HSL and 3-hydroxy-C10HSL are probably not essential for the expression of *B. pseudomallei* virulence and virulence-associated factors (3).

As in the *bpeAB* mutant, KHW carrying a plasmid that overproduces BpeR also synthesized less C8HSL than did the wild type, but it failed to produce any C10HSL, 3-hydroxy-C8HSL, or 3-oxo-C14HSL (Fig. 4B). This again lends support to the suggestion that the BpsIR system, which produces C8HSL, may be a key player in *B. pseudomallei* quorum sensing. While the roles of efflux pumps in the export of acyl-HSLs in *P. aeruginosa* have been described previously, the situation in *B. pseudomallei* appears to be unique because of the absolute requirement of BpeAB-OprB for extracellular secretion of all the different acyl-HSLs. We have thus identified a nonantibiotic physiological substrate of a multidrug efflux pump that is relevant to *B. pseudomallei* virulence. The consequence of defective BpeAB-OprB function is the complete inhibition of quorum sensing in *B. pseudomallei*, resulting in dramatic attenuation in virulence (3). Although the focus of this study was the BpeAB-OprB pump, it would also be prudent to study the roles of the other *B. pseudomallei* resistance-nodulation-division efflux pumps in the efflux of acyl-HSLs. In *P. aeruginosa*, reduced synthesis of 3-oxo-C12HSL and *N*-butanoyl-homoserine lactone (C4HSL), respectively, was associated with overexpression of the MexAB-OprM and MexEF-OprN efflux pumps (7, 10), while reduced production of quorum-sensing-regulated products was associated with an impaired MexGHI-OpmD pump (1). In contrast, the mechanism by which acyl-HSLs enter cells remains unknown, although it is widely believed to occur by passive diffusion.

Inhibition of BpeAB-OprB would be therapeutically beneficial, because it enhances the susceptibility of *B. pseudomallei* to the commonly used aminoglycoside and macrolide antibiotics and attenuates virulence by preventing quorum sensing. We have previously shown that the addition of C8HSL and C10HSL to mammalian cell cultures was able to restore the invasiveness and cytotoxicity of the *bpeAB* mutant to wild-type levels (3). Future work should include the identification of inhibitors and quenchers of quorum sensing, as well as inhibitors of BpeAB-OprB, and their evaluation as potential therapeutic agents against *B. pseudomallei* and other clinically relevant gram-negative bacterial pathogens.

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