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The BpsIR Quorum-Sensing System of *Burkholderia pseudomallei*

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BpsIR, a LuxIR quorum-sensing homolog, is required for optimal expression of virulence and secretion of exoproducts in *Burkholderia pseudomallei*. Cell density-dependent expression of *bpsI* and *bpsR*, the positive regulation of *bpsIR* expression by BpsR, and the synthesis of *N*-octanoyl-homoserine lactone (C8HSL) by BpsI are described in this report.

Quorum sensing regulates many diverse biological functions in gram-negative bacteria, including conjugation (20, 32), antibiotic synthesis, extracellular enzyme and exopolysaccharide production (13, 21), expression of extracellular virulence factors, and biofilm formation (5, 18). In this study, we describe BpsIR, a LuxIR homolog in *Burkholderia pseudomallei*, a gram-negative soil bacillus and etiologic agent of human and animal melioidosis.

Autoinducer synthesis by BpsIR in *B. pseudomallei* KHW. The bacterial strains and plasmids used in this study are described in Table 1. KHW*bpsI*::Km and KHW*bpsR*::Km insertion mutants were derived from a local virulent isolate, *B. pseudomallei* KHW, by gene replacement using the suicide vector pJQ200mp18 as described previously (2, 3). The *bpsI* and *bpsR* fragments were amplified from *B. pseudomallei* KHW genomic DNA using the primer pairs BpsIF (5′ATCTGCAG ATGCGAACTTTCGTTCATGGC) and BpsIR (5′ATCTGC AGGAAATACCGTTGAATGGTCCA) and BpsRF (5′ATC TGCAGATGGAACTGCGCTGGCAAGA) and BpsRR (5′A TCTGCAGTTACGGCGCGTCGATGAGCC), respectively (Fig. 1). Located on chromosome 2 of *B. pseudomallei*, *bpsIR* is highly similar to *pmlIR*, which was recently described by Valade et al. (29), and the BpsI and BpsR proteins are 75 and 80% identical to *B. cepacia* CepI and CepR, respectively (29). *bpsI* and *bpsR* are divergently transcribed, and the intergenic 742-bp spacer region contained two *lux* box motifs composed of 20-bp palindromic sequences which matched the consensus *lux* box in 15 and 13 of 20 positions, respectively (Fig. 1, insert) (10).

The promoter$_{bpsI}$-lacZ fusion (pSYI) and the promoter$_{bpsR}$-lacZ fusion (pSYR), obtained by ligating the putative *bpsI* and *bpsR* promoters, respectively, to *lacZ* on pCYY, were introduced into the wild type and *bpsIR* mutants to study transcriptional regulation of *bpsI* and *bpsR*. Exogenous addition of only 0.125 nM *N*-octanoyl-homoserine lactone (C8HSL) to KHW*bpsI*::Km (pSYI) restored the *bpsI* promoter activity to the wild-type level. The *bpsI* promoter was, in contrast, 10- and 1,600-fold less sensitive to C10HSL (*N*-decanoyl-L-homoserine lactone) and C6HSL (*N*-hexanoyl-L-homoserine lactone), respectively, and was insensitive to C4HSL (*N*-butyryl-L-homoserine lactone), 3OC6HSL [*N*-(3-oxohexanoyl)-L-homoserine lactone], 3OC8HSL [*N*-(3-oxooctanoyl)-L-homoserine lactone], 3OC10HSL [*N*-(3-oxodecanoyl)-L-homoserine lactone], and 3OC12HSL [*N*-(3-oxododecanoyl)-L-homoserine lactone] tested at concentrations up to 1 μM (data not shown).

Acyl-HSLs, extracted from the spent culture supernatant of *Escherichia coli* DH5α(pGEM-T-*bpsIR*) expressing *bpsI*, were analyzed by high-performance liquid chromatography (HPLC) using a C_{18} reversed-phase column (Agilent Series 1100 Hypersil octyldecyl silane column; 200 by 4.6 mm; particle size, 5 μm). Upon 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 15 min, and an isocratic profile over 25 min, the amount of acyl-HSLs in each fraction was quantified by the β-galactosidase activity produced using KHW*bpsI*::Km (pSYI). BpsI synthesized mainly C8HSL (Fig. 2). The PmlIR system, in comparison, synthesized predominantly C10HSL, but this may be attributed to the different *B. pseudomallei* strains used (29).

Cell density-dependent expression and transcriptional regulation of *bpsI* and *bpsR*. Cell density-dependent expression of *bpsI* was observed in the wild type, but not the *bpsI* or *bpsR* mutants, which is a characteristic of quorum-sensing genes (Fig. 3A and B). The addition of 0.125 nM C8HSL to the KHW*bpsI*::Km (pSYI) culture restored the cell density-dependent expression of *bpsI* previously absent in the *bpsI* mutant (Fig. 3A). The low-level expression of *bpsI* in KHW*bpsI*::Km suggests the possibility of residual autoinducers produced by other *B. pseudomallei* LuxIR homologs acting on the *bpsI* promoter (Fig. 3A and B). Like *bpsI*, transcription of *bpsR* was also cell density dependent (Fig. 3C) and was positively regulated by *bpsI* as well as its own gene product (Fig. 3C and D). Such findings are consistent with a model wherein the product of the BpsI synthase, an acyl-HSL, interacts with the BpsR, an acyl-HSL receptor protein, to activate the transcription of both *bpsI* and *bpsR* via interaction with the respective *lux* box motifs identified in the intergenic region between *bpsI* and *bpsR* (Fig. 1).

***bpsI* and *bpsR* mutants are partially attenuated in virulence in the *Caenorhabditis elegans* model.** In the *B. pseudomallei-C. elegans* coculture assay using synchronized L2-stage worms, twice as many worms survived after 48 h of coculture

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Reference or source
B. pseudomallei strains		
KHW	Wild-type, virulent clinical isolate	3
KHW*bpsI*::Km	Isogenic to KHW containing *bpsI*::Km	This study
KHW*bpsR*::Km	Isogenic to KHW containing *bpsR*::Km	This study
KHW*bpsI*::Km (pUCP28T-*bpsI*)	KHW*bpsI*::Km mutant complemented in *trans* with pUCP28T carrying full-length *bpsI* gene	This study
KHW *bpsR*::Km (pUCP28T-*bpsR*)	KHW*bpsR*::Km mutant complemented in *trans* with pUCP28T carrying full-length *bpsR* gene	This study
KHW(pSYI)	KHW carrying the plasmid pSYI; Tc^r	This study
KHW(pSYR)	KHW carrying the plasmid pSYR; Tc^r	This study
E. coli strains		
DH5αλpir	DH5α with a λ prophage carrying the gene encoding the p protein; Km^s Tp^s Gm^s	17
HB101(pRK600)	Helper strain; containing pRK600 for triparental mating, *supE44 hsdS20*(r_Bm_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*; Cm^r	7, 23
SM10	Mobilizing strain; RP4 *tra* genes integrated in chromosome; Km^r	24
Plasmids		
pGEM-T	Vector for PCR cloning; Ap^r	Promega
pUTKm	Source of kanamycin resistance cassette; oriR6K mobRP4; Km^r Ap^r	6
pUCP28T	Broad-host-range vector; IncP OriT; pRO1600 ori; Tp^r	30
pJQ200mp18	Mobilizable allelic exchange vector; *traJ sacB* Gm^r	22
Mini-CTX1	Mobilizable, broad-host-range plasmid for engineering of reporter strains; *oriT* Tc^r	11
pMC1403	pBR322 derivative carrying a 6.2-kb promoterless *lacZYA* fragment for the cloning of translational control signals and 5′ coding sequences of exogenously derived genes; Ap^r	1
pCYY	Mini-CTX1 containing a 6.2-kb promoterless *lacZYA* fragment inserted into EcoRI-SalI site in the multiple cloning sites of pMC1403	This study
pSYI	pCYY containing a 1.2-kb *bpsI′-lacZ*$^+$ gene fusion; Tc^r	This study
pSYR	pCYY containing a 1.2-kb *bpsR′-lacZ*$^+$ gene fusion; Tc^r	This study
pGEM-T-*bpsI*::Km	pGEM-T carrying 710-bp *bpsI* with a 2.3-kb kanamycin resistance cassette inserted at a blunt-ended BamHI site 330 bp from the start of the *bpsI* coding sequence; Ap^r Km^r	This study
pGEM-T-*bpsIR*	pGEM-T carrying the full-length 2,271-bp *bpsIR* fragment for heterologous expression in *E. coli*	This study
pJQ*bpsI*::Km	pJQ200mp18 with a 3.0-kb PstI fragment from pGEMT-*bpsI*::Km containing *bpsI*::Km; Gm^r Km^r	This study
pJQ*bpsR*::Km	pJQ200mp18 carrying *bpsR*::Km; Gm^r Km^r. The 2.3-kb kanamycin resistance cassette from pUTKm was made blunt ended and inserted into the blunt-ended AatII site, 410 bp from the start of the coding sequence of *bpsR*	This study

with KHW*bpsI*::Km as with KHW and the complemented KHW*bpsI*::Km mutant (Fig. 4A) (8). Similarly, twice as many worms survived when fed on KHW*bpsR*::Km as when fed on KHW and the complemented KHW*bpsR*::Km mutant (Fig. 4B). The partial attenuation in *C. elegans* killing observed with the *bpsI* and *bpsR* mutants may be attributed to the presence of other *luxIR* homologs which may interact to control virulence in *B. pseudomallei* as in the cases of *Vibrio cholera* and *Pseudomonas aeruginosa* (16, 19). Two other *luxIR* homologs have been identified in the recently sequenced *B. pseudomallei* K96243 genome. In *P. aeruginosa*, the quorum-sensing mutants were also not completely avirulent in both mammalian models of infection and pathogen-*C. elegans* coculture assay (25). The expression of *B. pseudomallei* virulence is probably multifactorial, and although quorum-sensing genes have significant effects on virulence, many other factors also play important roles in regulating pathogenesis.

BpsIR is involved in the secretion of some exoproducts. Previous studies have demonstrated that *B. pseudomallei* secreted protease, lipase, and phospholipase C (PLC) into the extracellular milieu, but the roles of these in pathogenesis have not been elucidated. Siderophore production in *B. pseudomallei* KHW was growth phase dependent, with maximal siderophore production occurring in the stationary-phase culture supernatants (data not shown). Both the *bpsI* and *bpsR* mutants yielded two- to threefold more siderophores than the wild type (Fig. 5A). Complementation of the mutants by use of the plasmids pUCP28T-*bpsI* and pUCP28T-*bpsR* restored the siderophores' levels to those of the wild type (Fig. 5A). Siderophores, which function in the sequestration of iron, are implicated in the virulence of several pathogenic bacteria, including *B. cepacia*, where the CepIR quorum-sensing system also negatively regulates ornibactin synthesis, but the significance of such a mechanism is also not established (14).

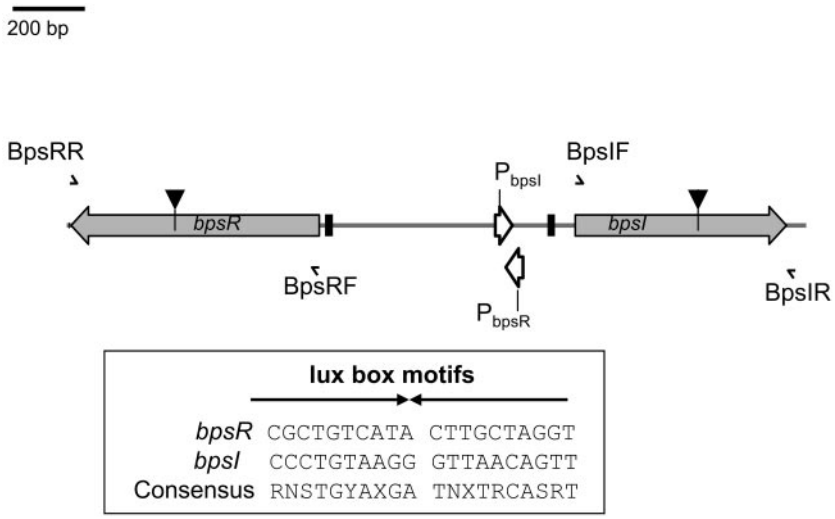


FIG. 1. Genetic organization of the *bpsI-bpsR* locus of *B. pseudomallei* KHW (GenBank accession no. AY373337). Gray box arrows denote the divergently transcribed *bpsI* and *bpsR* genes; short thin arrows denote the PCR primer positions, while short white box arrows denote putative *bpsI* and *bpsR* promoters (P). Triangles indicate the locations where kanamycin resistance cassettes were inserted in the null mutants, KHW*bpsI*::Km and KHW*bpsR*::Km. The insertion sites were confirmed by PCR and DNA sequencing, while the null phenotypes were confirmed by Northern blotting (data not shown). Black rectangles denote the *lux* boxes, and their similarities with the palindromic *lux* box consensus sequence are shown in the insert (10). Consensus sequence abbreviations are as follows: N is A, T, C, or G; R is A or G; S is C or G; Y is T or C; and X is N or a gap in the sequence. The length of the bar represents a distance of 200 bp.

PLC production in the culture supernatants of *B. pseudomallei* was also dependent on the growth phase, with maximal production of PLC occurring at the late log phase (data not shown). Unlike siderophore production, PLC production in *B. pseudomallei* is positively regulated by the BpsIR quorum-sensing system, and the production of PLC in the supernatants of 24-h-old cultures was reduced to half in the *bpsI* and *bpsR* mutants compared to that in the wild type (Fig. 5B). The production of PLC was likewise restored to wild-type levels in the *trans*-complemented KHW*bpsI*::Km and KHW*bpsR*::Km mutants (Fig. 5B). The twofold difference between the wild type and the mutants suggests either that the PLC promoter may be indirectly controlled by the BpsIR quorum-sensing system or, alternatively, that the high basal level of PLC expression in the wild type and mutants might be attributable to a second acyl-HSL system which bears upon the PLC promoter. Since PLC is believed to be important for interaction with the phospholipids in eukaryotic cell membranes during

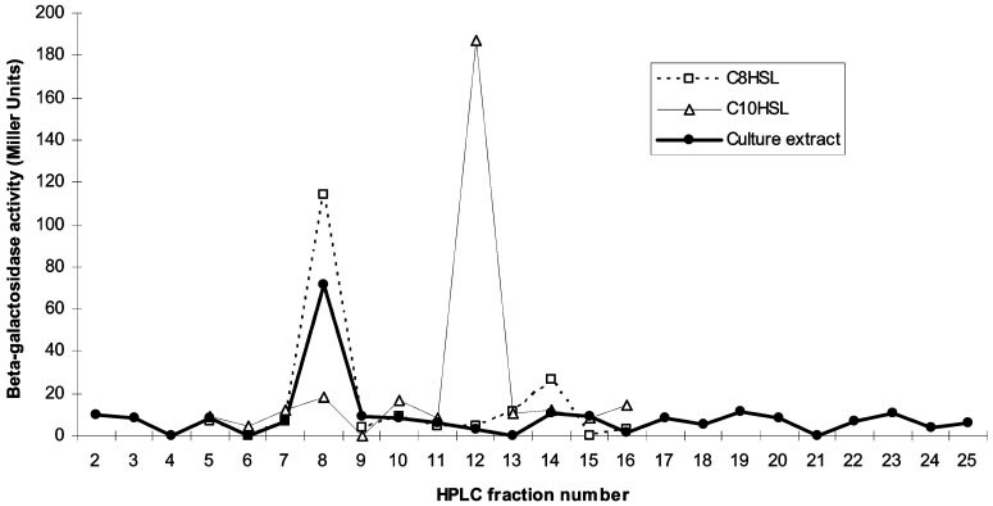
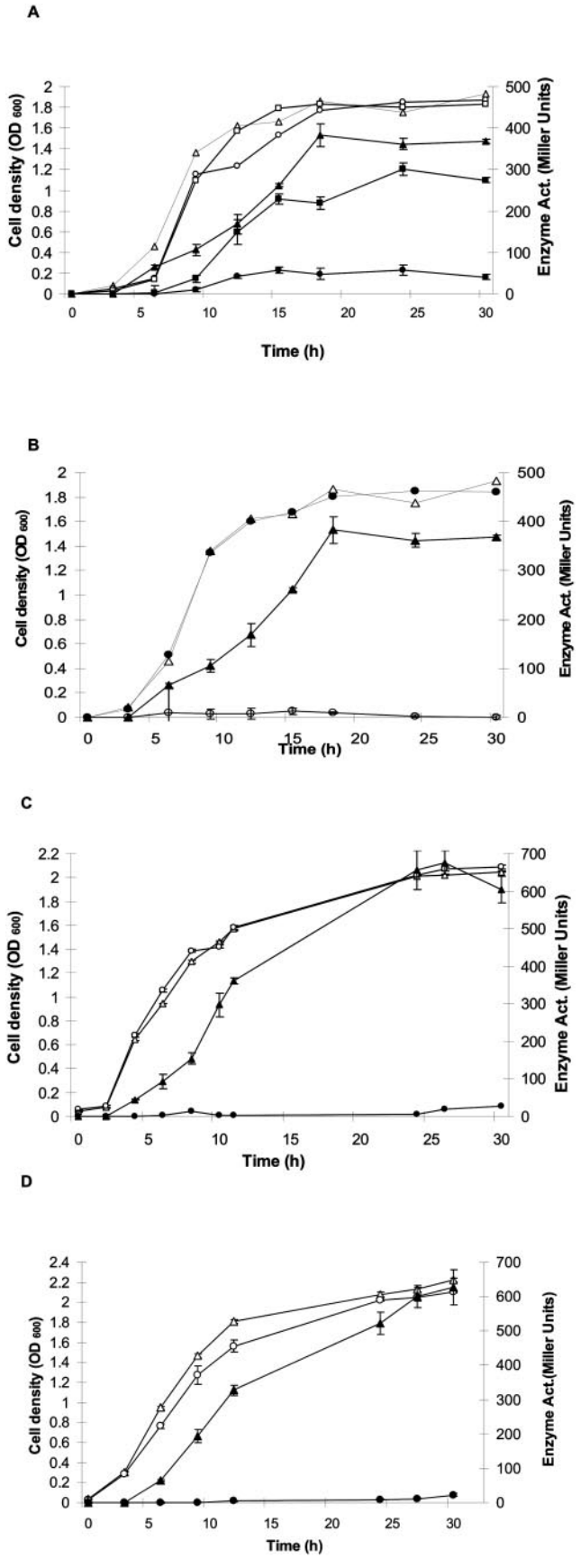


FIG. 2. Detection of acyl-HSLs produced by BpsI using HPLC. The culture extract (filled circles) contained acyl-HSL extracted from the supernatant of a stationary-phase culture of *E. coli* DH5α (pGEM-T-*bpsIR*) in AB medium (4) supplemented with 0.1 μg of thiamine/ml, 0.3% Casamino Acids, and 20 mM glycerol. Concentrated extract was chromatographed on a C_{18} reversed-phase HPLC column. Each 2-ml fraction collected was concentrated and assayed for β-galactosidase activity using KHW*bpsI*::Km (pSYI) as the reporter strain and according to the method described by Miller (15). For the profiles of the synthetic C8HSL (open squares) and C10HSL (open triangles) standards, only fractions 5 to 16 are represented, since the others did not yield any detectable activity.



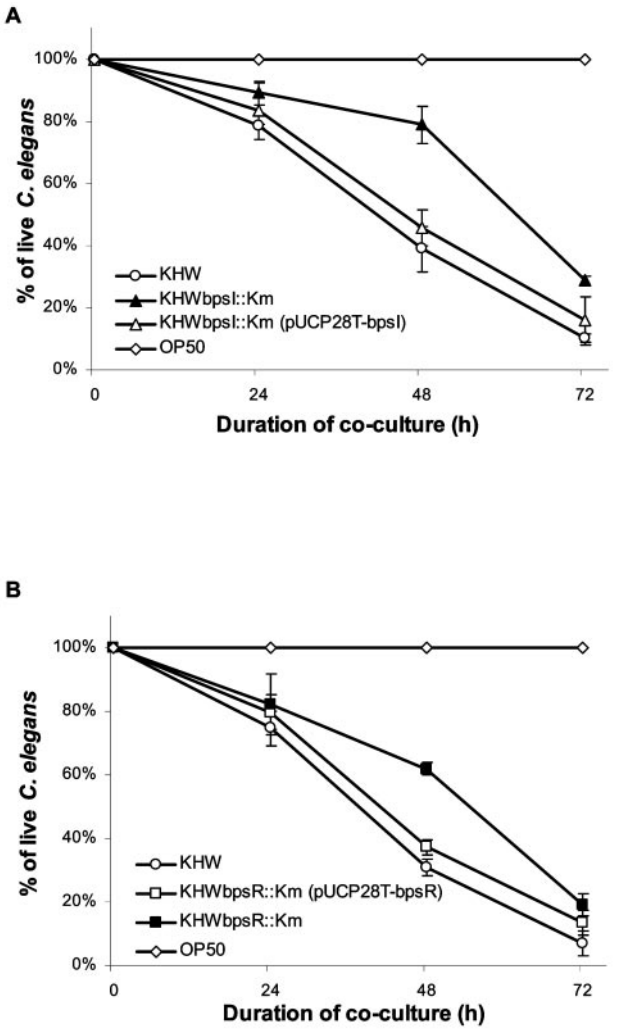


FIG. 4. Effects of *bpsI* and *bpsR* mutations on *B. pseudomallei* virulence. Both KHW*bpsI*::Km and KHW*bspR*::Km were attenuated in virulence in the *B. pseudomallei-C. elegans* coculture assays. After 48 h of coculture, 79% of the worms fed on KHW*bpsI*::Km survived, compared to 39 and 46% survival rates for those worms fed on KHW and the complemented KHW*bpsI*::Km strains, respectively (A). KHW*bpsR*::Km was also attenuated in virulence with 62% of the worms surviving after 48 h of coculture, compared to 31 and 37% survival rates in the wild-type KHW and complemented KHW*bpsR*::Km strains, respectively (B). *E. coli* OP50 was used as a negative control in this assay.

FIG. 3. Cell density-dependent expressions of *bpsI* and *bpsR*. Open symbols represent cell densities, while filled symbols represent β-galactosidase activities. Cell densities and β-galactosidase activities of KHW(pSYI) and KHW(pSYR) are represented as open and filled triangles, respectively. Cell density-dependent expression of *bpsI* was abolished in KHW*bpsI*::Km(pSYI) (circles, A), and KHW*bpsR*::Km(pSYI) (circles, B). Addition of exogenous 0.125 nM C8HSL to KHW*bpsR*::Km(pSYI) did not affect the growth curve (open squares, A) but restored *bpsI* expression to almost wild-type levels (filled squares, A). KHW did not express any endogenous β-galactosidase activity (data not shown). Cell density-dependent expression of *bpsR* was also abolished in KHW*bpsI*::Km(pSYR) (circles, C) and KHW*bpsR*::Km(pSYR) (circles, D). β-Galactosidase activities were expressed in Miller units. The experiments were performed in triplicate. OD_{600}, optical density at 600 nm.

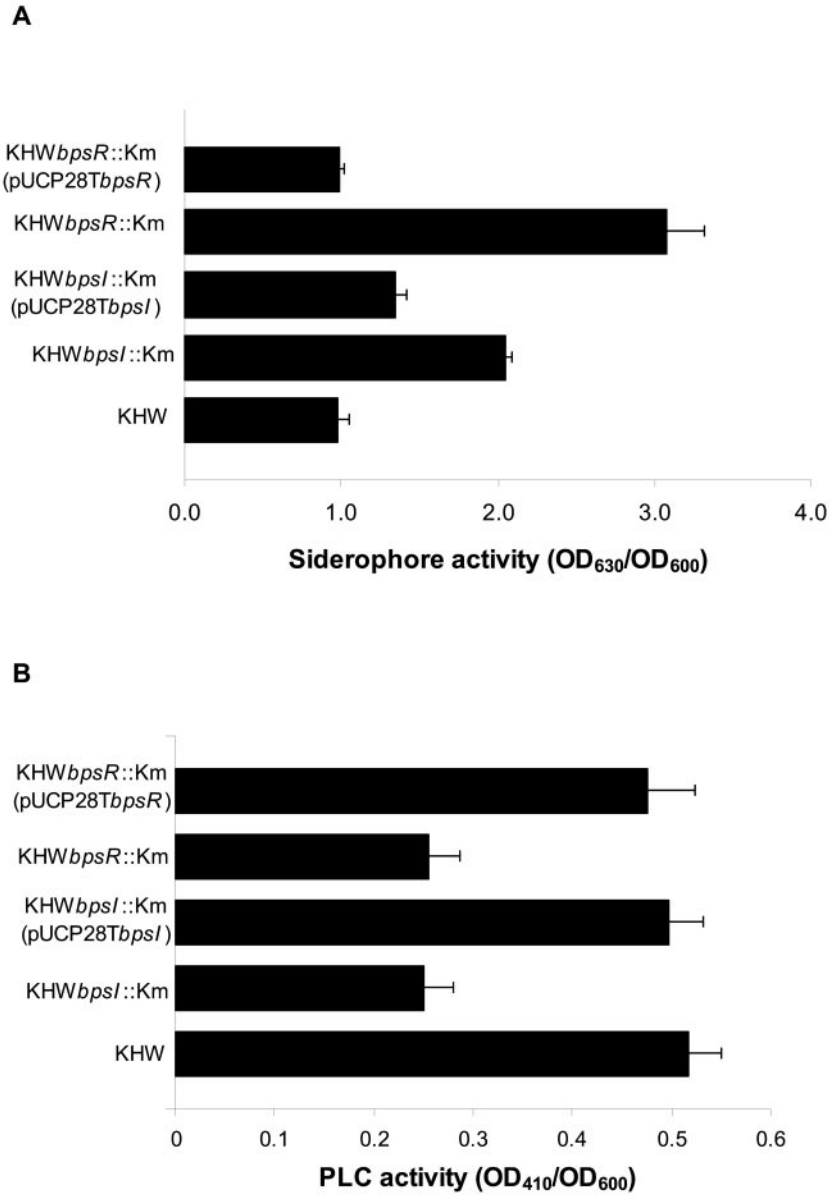


FIG. 5. (A) Siderophore secretion in *B. pseudomallei* KHW was negatively regulated by the BpsIR quorum-sensing system. Siderophore activities were assayed in the 24-h-old culture supernatants of *B. pseudomallei* of KHW, KHW*bpsI*::Km, KHW*bpsI*::Km(pUCP28T-*bpsI*), KHW*bpsR*::Km, and KHW*bpsR*::Km(pUCP28T-*bpsR*) by measuring the differential in readings of optical density at 630 nm (OD_{630}) between the test and the sample blank using the chrome azurol S assay as described in Yang et al. (31). The values shown have been normalized for cell density by expressing them as a ratio of $\Delta OD_{630}/OD_{600}$. Each bar represents the average (± standard deviation) of readings from three independent experiments. (B) PLC secretion by *B. pseudomallei* KHW is positively regulated by the BpsIR quorum-sensing system. PLC activities were determined in the supernatants of 24-h cultures of *B. pseudomallei* KHW, KHW*bpsI*::Km, KHW*bpsI*::Km(pUCP28T-*bpsI*), KHW*bpsR*::Km, and KHW*bpsR*::Km(pUCP28T-*bpsR*) by the method described by Kurioka et al. (12). The data presented are the averages (± standard deviations) of the results from three independent experiments.

infection, the former suggestion would explain how its production is positively regulated by quorum sensing (27, 28).

The secretion of lipase by *B. pseudomallei* KHW, which was also growth phase dependent, was unaffected in the *bpsIR* mutants (data not shown). Likewise, protease secretion in *B. pseudomallei* KHW, as detected by a zone of clearance around the colonies on dialyzed brain heart infusion agar supplemented with 1.5% skim milk (26), was also unaffected by the *bpsIR* mutations (data not shown). It is also unclear if protease is a virulence determinant in *B. pseudomallei*, since there was no correlation between virulence and the level of exoproteolytic activity when *B. pseudomallei* was injected into mice via the intraperitoneal route (9). Further studies are needed to determine the mechanisms by which BpsIR regulates the production of siderophores and phospholipase C in *B. pseudomallei*.

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