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Elucidation of the Regulon and *cis*-Acting Regulatory Element of HrpB, the AraC-Type Regulator of a Plant Pathogen-Like Type III Secretion System in *Burkholderia pseudomallei*[▽]†

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The human pathogen *Burkholderia pseudomallei* possesses multiple type III secretion system (T3SS) gene clusters. One of these, the *B. pseudomallei* T3SS2 ($T3SS2_{bp}$) gene cluster, which apparently plays no role in animal virulence, is also found in six additional *Burkholderia* spp. and is very similar to T3SSs found in phytopathogenic *Xanthomonas* spp. and *Ralstonia solanacearum*. The $T3SS2_{bp}$ gene cluster also encodes an AraC-type regulatory protein ($HrpB_{bp}$) that is an ortholog of HrpB, the master regulator of the *R. solanacearum* T3SS ($T3SS_{rso}$) and its secreted effectors. Transcriptome analysis showed that $HrpB_{bp}$ activates the expression of $T3SS2_{bp}$ genes, as well as their orthologs in *R. solanacearum*. In addition to activating $T3SS2_{bp}$, $HrpB_{bp}$ also upregulates the expression of ~30 additional *B. pseudomallei* genes, including some that may confer production of adhesive pili, a polyketide toxin, several putative $T3SS2_{bp}$-secreted effectors, and components of a regulatory cascade. $T3SS2_{bp}$ promoter regions were found to contain a conserved DNA motif ($p2_{bp}$ box) identical in sequence and position to the hrp_{II} box required for HrpB-dependent $T3SS_{rso}$ transcription activation. The $p2_{bp}$ box is also present in the promoter regions of the essentially identical T3SS found in the very closely related species *Burkholderia thailandensis* ($T3SS2_{bt}$). Analysis of $p2_{bp}$ box mutants showed that it is essential for $HrpB_{bp}$-mediated transcription activation in both species. Although it has been suggested that $T3SS2_{bp}$ and $T3SS2_{bt}$ may function in phytopathogenicity, we were unable to demonstrate a phytopathogenic phenotype for *B. thailandensis* in three different plant hosts.

Burkholderia pseudomallei is a pathogenic betaproteobacterium primarily found in wet soil and water in southeast Asia and northern Australia (32, 75). Infection with *B. pseudomallei* by inhalation or through open wounds results in melioidosis, a potentially deadly human disease predominately affecting individuals with preexisting conditions, such as diabetes or renal disease (14). Melioidosis is characterized by septicemia, pneumonia, and pulmonary, splenic, and/or hepatic abscesses (14, 75). No vaccine for this disease is available, and the disease is also difficult to diagnose and treat (75). The CDC lists *B. pseudomallei* as a category B select agent restricted to biosafety level 3 (BSL3) containment (54), underscoring the threat posed by this pathogen.

Type III secretion systems (T3SSs) are specialized macromolecular machines essential for virulence of many Gram-negative animal- and plant-pathogenic bacteria. T3SSs are comprised of 20 to 25 proteins, 9 of which are conserved among all known T3SSs (19). T3SSs deliver proteins, termed effectors, into the cytoplasm of eukaryotic cells via a structure variously termed a needle, filament, or pilus (19). Effector functions vary widely and include altering signal transduction, transcriptional activities, and protein turnover in host cells (2, 6).

The large (~8-Mb) genome of *B. pseudomallei* encodes three T3SSs, compared to only 1 encoded by most other pathogenic bacteria (29). One of these, T3SS3, is orthologous to the Inv/Mxi-Spa T3SSs in *Salmonella* and *Shigella* spp. and is required for virulence in mice and hamsters (64, 71). The other two (T3SS1 and *B. pseudomallei* T3SS2 [$T3SS2_{bp}$]) show similarity to the T3SS found in the plant pathogen *Ralstonia solanacearum* ($T3SS_{rso}$), and neither is involved in animal virulence (52, 71). T3SS1 is unique to *B. pseudomallei*, while $T3SS2_{bp}$, the focus of this study, is also found in *Burkholderia thailandensis*, a very closely related soil-dwelling bacterium from Thailand which was initially classified as *B. pseudomallei*. *B. thailandensis* is capable of causing disease in animal models, although its 50% lethal dose (LD_{50}) is at least 10^5-fold higher than that of *B. pseudomallei* (7, 8). Since *B. thailandensis* apparently does not cause disease in humans, it is used as a surrogate for *B. pseudomallei* infection (28, 74).

$T3SS_{rso}$ in *R. solanacearum*, like $T3SS2_{bp}$, has an AraC-type transcriptional regulator (HrpB) as part of the T3SS cluster. HrpB is the master regulator of transcription of $T3SS_{rso}$ and its secreted effectors. It binds to a conserved DNA motif called the hrp_{II} box that is located 46 bp upstream of the transcriptional start of the $T3SS_{rso}$ operons or effector genes it regulates (21). *Xanthomonas* spp., which are also plant pathogens, have a very similar AraC-type transcriptional regulator, HrpX, which regulates T3SS transcription via a nearly identical DNA motif called the PIP box (73). Expression of HrpB in *R. solanacearum* is induced by either growth in minimal medium (3) or plant-cell contact (41). Induction via plant-cell contact is mediated by a regulatory cascade in which an unknown signal is sensed by the outer membrane receptor protein PrhA and transduced through the transmembrane protein PrhR, which in turn activates the extracytoplasmic sigma factor PrhI (1, 9).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference and/or source
Strains		
B. pseudomallei DD503	Δ(*amrR-oprA*) *rpsL* (Sm^r) Km^s	43
B. thailandensis DW503	Δ(*amrR-oprA*) *rpsL* (Sm^r) Km^s Cm^s Gm^s	11
B. thailandensis LL102	Derivative of DW503, $hrpB_{bt}$::pGHrpBm Gm^r	This study
B. thailandensis E264	Wild type	7
R. solanacearum GMI1000	Wild type	56
P. putida ATCC 12633	Wild type	63
E. coli NEB5-alpha	Cloning strain	Invitrogen
E. coli S17-1	Tra^+ Pm^s	62
E. coli HB101(pRK2013)	Tra^+ mobilization strain, Pm^s Km^r	26
Plasmids		
pCR2.1-TOPO	Cloning vector, Km^r Amp^r	Invitrogen
pCR-XL-TOPO	Cloning vector, Km^r Zeo^r	Invitrogen
pSCRhaB2K	Broad-host-range expression vector derived from pSCRhaB2, Km^r	W. Nierman, reference 12
pPR9TT	Vector with promoterless *lacZ*, Cm^r Amp^r	58
pGSV3	Suicide vector, Gm^r	24
pGHrpBm	406-bp internal fragment of *hrpB* in pGSV3, Gm^r	This study
pSCR1610a	−160 bp relative to ATG start codon to +31 bp relative to TGA stop codon of BPSS1610 in pSCRhaB2K, Km^r	This study
pPR1606	−356 to +62 bp relative to ATG start codon of BPSS1606 in pPR9TT, Cm^r	This study
pPR0764	−279 to +62 bp relative to ATG start codon of BTH_II0764 in pPR9TT, Cm^r	This study
pPR0764Md	−160 to +62 bp relative to ATG of BTH_II0764 in pPR9TT, Cm^r	This study
pPR0764Tr	−114 to +62 bp relative to ATG of BTH_II0764 in pPR9TT, Cm^r	This study
pPR0764A2[b]	pPR0764 with T to A at −48 and −49, Cm^r	This study
pPR0764B2[b]	pPR0764 with T to A at −68 and −69, Cm^r	This study
pPR0764C2[b]	pPR0764 with T to A at −48, −49, −68, and −69, Cm^r	This study
pPR07641a[b]	pPR0764 with C to A at −67, Cm^r	This study
pPR07648a[b]	pPR0764 with G to T at −65, G to A at −62, and G to T at −51, Cm^r	This study

[a] Km, kanamycin; Gm, gentamicin; Pm, polymyxin B; Cm, chloramphenicol; Zeo, Zeocin; Amp, ampicillin; Sm, streptomycin; S, sensitive; R, resistant.
[b] For pPR0764 derivatives, numbers refer to nucleotide positions relative to the BTH_II0764 transcription start.

PrhI is required for expression of *prhJ* (9). PrhJ is a LuxR/UhpA-type regulator that activates transcription of the response regulator *hrpG*, the product of which activates expression of *hrpB* (9).

To investigate the significance and role of $T3SS2_{bp}$ in *B. pseudomallei*, we explored its regulation by the HrpB ortholog $HrpB_{bp}$. We found that $HrpB_{bp}$ activates $T3SS2_{bp}$ gene expression in a manner analogous to HrpB activation of T3SS expression in *R. solanacearum*, utilizing a DNA motif identical in sequence and position to the hrp_{II} box utilized by HrpB in *R. solanacearum*. We also defined the $HrpB_{bp}$ regulon in *B. pseudomallei* by identifying ~30 additional genes whose expression is $HrpB_{bp}$ activated.

MATERIALS AND METHODS

Growth conditions. Bacterial strains and plasmids used are listed in Table 1. *Escherichia coli*, *B. pseudomallei*, and *B. thailandensis* were grown at 37°C in Luria-Bertani medium (LB) (42), LB supplemented with 2% glycerol (LBG), or M9 medium (57) with 20 mM glutamate instead of glucose. *R. solanacearum* and *Pseudomonas putida* were grown at 30°C in 1% Bacto peptone, 0.1% Casamino Acids, 0.1% yeast extract, and 0.5% glucose (BG) (36). When necessary, antibiotics were used at the following concentrations: kanamycin at 50 μg/ml (Km_{50}) for *Burkholderia* spp. and 25 μg/ml (Km_{25}) for *R. solanacearum*; 20 μg/ml polymyxin B (Pm_{20}), 20 μg/ml gentamicin (Gm_{20}), and chloramphenicol at 20 μg/ml (Cm_{20}) for *E. coli* and 50 μg/ml (Cm_{50}) for *B. thailandensis*.

Plasmid construction. (i) pSCR1610a. Primers 1610F and 1610R (see Table S1 in the supplemental material) were used to PCR amplify the BPSS1610 coding sequence (CDS) ($hrpB_{bp}$), which was then cloned into pCR2.1-TOPO. This plasmid was digested with XbaI and HindIII, and the resultant fragment was ligated into similarly digested pSCRhaB2K. The accuracy of the cloned BPSS1610 DNA CDS was confirmed by DNA sequencing.

(ii) pGHrpBm. A 406-bp internal fragment of the BTH_II0762 CDS (*B. thailandensis hrpB* [$hrpB_{bt}$]) was PCR amplified with HrpBmF and HrpBmR primers and cloned into pCRXL-TOPO. A SpeI-NotI fragment from this plasmid was ligated into the similarly digested suicide vector pGSV3.

(iii) pPR1606, pPR0764, pPR0764Md, and pPR0764Tr. The promoter regions of BTH_II0764 and BPSS1606 were PCR amplified with PromFLF/PromR and 1606PF/1606PR primers, respectively, and individually cloned into pCR2.1-TOPO. PstI-XhoI fragments from each plasmid were cloned into the similarly digested promoterless *lacZ* fusion vector pPR9TT, yielding pPR0764 and pPR1606. Similarly, two shorter promoter region fragments of BTH_II0764 were PCR amplified using primers PromMDF/PromR and PromTrF/PromR and individually cloned into pCR2.1-TOPO. PstI-XhoI fragments from each plasmid were cloned into similarly digested pPR9TT, to yield pPR0764Md and pPR0764Tr, respectively.

(iv) pPR0764A2, pPR0764B2, and pPR0764C2. Splice overlap extension PCR (30) with primers incorporating specific nucleotide changes was used to introduce site-specific mutations into the promoter region of BTH_II0764. Briefly, PromFLF/MutA2 and MutA3/PromR primers were used to generate two overlapping amplicons that were gel purified and used in a second PCR with only the PromFL/PromR primers. This amplicon was cloned into pCR2.1-TOPO. The resultant plasmid was digested with PstI and XhoI and ligated into similarly digested pPR9TT, yielding pPR0764A2. This procedure was repeated using the PromFLF/MutB2 and MutB3/PromR primers for the construction of pPR0764B2 and the PromFLF/MutC2 and MutC3/PromR primers for pPR0764C2.

(v) pPR07641a and pPR07648a. Error-prone PCR with PromFLF/PromR primers was used to enhance misincorporation of nucleotides in the promoter fragment of BTH_II0764. To do this, 0.5 mM $MnCl_2$ was added to the PCR and the dCTP and dTTP concentrations were changed to 0.55 mM each. The resultant amplicon was gel purified, digested with PstI and XhoI, ligated into similarly

digested pPR9TT, and transformed into *E. coli* NEB5-alpha cells. All pPR9TT plasmid inserts were subjected to DNA sequencing.

All DNA sequences were obtained from Integrated Microbial Genomes (IMG; http://img.jgi.doe.gov). PCR conditions were 98°C for 4 min, 30 cycles of 98°C for 30 s, 60°C for 30 s, and 72°C for 1 min (except for the case of BPSS1610, for which it was 2 min), and a final extension of 72°C for 10 min.

Strain construction. Chemically competent *E. coli* NEB5-alpha cells and *E. coli* S17-1 cells (62) were transformed as previously described (61). The pSCRhaB2K- and pPR9TT-based plasmids were transferred from *E. coli* NEB5-alpha into *B. pseudomallei*, *B. thailandensis*, or *R. solanacearum* recipients by triparental mating. Briefly, donor, recipient, and *E. coli* HB101(pRK2013) helper were grown overnight on antibiotic selection plates, resuspended in LBG or BG to an optical density at 600 nm (OD_{600}) of 0.2, and shaken until the OD_{600} reached 0.4. Equal volumes of the cultures were mixed, and 30 μl was spotted onto LBG or BG plates. After 18 h, cells were resuspended in sterile water and spread onto selection plates containing the appropriate antibiotic to select for the desired plasmid and Pm_{20} to counterselect against the *E. coli* donor and helper strains.

To inactivate *$hrpB_{bt}$*, the suicide plasmid pGHrpBm (Table 1; see above) was transferred into *B. thailandensis* from *E. coli* S17-1, as described above, and integrants were selected on plates with Gm_{20} and Pm_{20}. Proper insertion of pGHrpBm into the genome of *B. thailandensis* was confirmed by PCR.

β-Galactosidase assays. Cells from overnight plate cultures were suspended in LB or M9–20 mM glutamate to an OD_{600} of 0.2 and shaken until the OD_{600} reached 0.4. If induction of $HrpB_{bp}$ overexpression was needed, rhamnose was added to 0.2% and the cells were harvested 3 h later; otherwise, the cells were harvested at an OD_{600} of 1.0. β-Galactosidase assays were performed as previously described (15) and repeated at least three times.

Quantitative reverse transcription PCR (qRT-PCR). Cells were grown overnight on plates, suspended in LBG or BG to an OD_{600} of 0.2, and shaken until the OD_{600} reached 0.6. Rhamnose was added to 0.2% to induce $HrpB_{bp}$ expression, and incubation continued until the OD_{600} was 1.2. After an equal volume of RNAprotect (Qiagen) was added to the culture, RNA was purified from pelleted cells by using the RNeasy kit (Qiagen) and treated with DNase I (Qiagen) according to manufacturer's instructions, except that the amount of DNase I was increased 3-fold. cDNA was synthesized using 1 μg of RNA and the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. Quantitative PCR was performed on 22 target genes with an Applied Biosystems StepOne real-time PCR system using Sybr green under the following conditions: 10 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 1 min. The amplification data were analyzed by the $2^{-\Delta\Delta C_T}$ method to determine relative $\log_2$ changes in gene expression (38) in reference to *rpoA* for *B. pseudomallei* and *B. thailandensis* and the cytochrome oxidase gene (RSc0369) for *R. solanacearum*. We chose *rpoA* as a reference because of its stable expression under a variety of conditions (53). Means and standard deviations were calculated from at least three independent experiments.

Whole-genome expression profiling. RNA from *B. pseudomallei* was prepared as described above. For microarray analysis, cDNA was synthesized, labeled with Cy3/Cy5, and hybridized to a whole-genome PCR amplicon microarray for *B. pseudomallei*, and raw data were analyzed as described previously (59). For massively parallel RNA sequencing (RNA-Seq) (76), the rRNA content was reduced by 2 rounds of treatment with MicrobExpress (Ambion). This mRNA-enriched fraction was used to synthesize cDNA by using SuperScript II (Invitrogen) with random hexamer primers and sequenced with a Genome Analyzer (Illumina Technologies). Data were analyzed by using the CLC Genomics workbench (CLC bio) allowing for 2 mismatches per read.

Determination of transcription start sites. A 5′ rapid amplification of cDNA ends (RACE) system (Invitrogen) was used according to the manufacturer's instructions. Briefly, the antisense primers for BTH_II0764 and BPSS1622 mRNA (RACEFL1 and S1622race1, respectively; see Table S1 in the supplemental material) were used to prime synthesis of cDNA to the 5′ end of the transcript. A poly(C) tail was then added to the 3′ end of the cDNA by using terminal deoxynucleotidyl transferase. Primer RACEFL2 or S1622raceb2 (for BTH_II0764 or BPSS1622, respectively) and a primer specific for the poly(C) tail (provided with the kit) were used to amplify the cDNA. The amplicons were then cloned into pCR2.1-TOPO and subjected to DNA sequencing.

Plant pathogenicity assays. Tomato seeds (*Solanum lycopersicum* var. *cerasiforme*) were surface sterilized with 15% bleach for 15 min, germinated, and grown for 4 weeks at 25°C on Murashige and Skoog medium (MS) solidified with 0.8% agar (46). Resultant plantlets were inoculated with bacteria as previously described (34). Briefly, plantlets with 2 or 3 leaves were placed in 50-ml Falcon tubes containing 5 ml of MS with 10^6 cells/ml of each test bacterium, and symptom development was monitored at 30°C for 7 days. Commercial tomato plants ("Better Boy") with 7 or 8 leaves were inoculated via the stub of a cut petiole (55) with a 2-μl cell suspension, and symptoms were monitored at 30°C for 7 days. *Arabidopsis thaliana* seeds (Col-0; Lehle Seeds) were incubated in moist *Arabidopsis* growing medium (Lehle Seeds) at 4°C for 2 days and then germinated at 25°C. Leaves of 3-week-old plants were infiltrated with each test bacterium, and disease was monitored as described previously (27).

To evaluate bacterial multiplication in plants, 0.3-cm^2 leaf discs from *A. thaliana* or stem sections from tomato were removed, flame sterilized, macerated or cut into ~1-mm sections, and agitated for 30 min at 25°C in sterile water. Serial dilutions of the supernatant were plated and colonies counted. Hypersensitive-response assays were performed on fully expanded leaves of *Nicotiana tabacum* (tobacco) by infiltration of each test bacterium into the leaves and monitored as previously described (31).

Microarray data accession number. The microarray data were deposited into the ArrayExpress database (49) under the accession number E-MTAB-484.

RESULTS

$T3SS2_{bp}$ is remarkably similar to the T3SSs in the phytopathogens *R. solanacearum* and *Xanthomonas* species. Nearly identical clusters of genes orthologous to the $T3SS2_{bp}$ cluster are found not only in *B. thailandensis* but also in *Burkholderia mallei*, *Burkholderia ubonensis*, *Burkholderia oklahomensis*, *Burkholderia ambifaria*, and *Burkholderia dolosa*. None of these are reported to be plant pathogens, and all except *B. mallei* and *B. dolosa* were isolated from soil. *Burkholderia* spp. that do not have a T3SS orthologous to $T3SS2_{bp}$ include *Burkholderia cenocepacia*, *Burkholderia multivorans*, *Burkholderia xenovorans*, *Burkholderia graminis*, *Burkholderia glumae*, *Burkholderia phytofirmans*, *Burkholderia phymatum*, *Burkholderia vietnamensis*, and *Burkholderia rhizoxinica*. Of the characterized plant pathogens, *R. solanacearum* and *Xanthomonas* spp., but not *Pseudomonas syringae* or *Erwinia* spp., have T3SSs that are also orthologous to $T3SS2_{bp}$.

Given the relationship of $T3SS2_{bp}$ to plant pathogen T3SSs, we examined the evolutionary relationships between the *Burkholderia* $T3SS2_{bp}$ orthologs and those in well-characterized plant pathogen T3SSs. A neighbor-joining phylogenetic tree was constructed using 3 of the 9 core T3SS genes that are conserved among all bacterial T3SSs (Fig. 1). Pairwise distances suggest that the orthologous T3SSs of *B. mallei*, *B. thailandensis*, *B. oklahomensis*, and *B. ubonensis* are the most closely related to $T3SS2_{bp}$ in *B. pseudomallei*, followed by the T3SSs of *B. ambifaria* and *B. dolosa*. The DNA sequence identities for *hrcV* orthologs are 99% between *B. pseudomallei* and *B. mallei*, ~94% between *B. pseudomallei*, *B. thailandensis*, *B. oklahomensis*, and *B. ubonensis*, and ~75% between *B. pseudomallei*, *B. ambifaria*, and *B. dolosa*, indicating a high degree of conservation. This example is illustrative of the DNA sequence identities between all the core T3SS genes among these *Burkholderia* spp. and largely reflects phylogenetic distance based on 16S rRNA sequence analysis (67). Of the well-characterized T3SSs in plant pathogens, the one in *R. solanacearum* ($T3SS_{rso}$) is the most closely related to $T3SS2_{bp}$.

Nineteen of the 20 genes in the $T3SS2_{bp}$ gene cluster have orthologs in the above-mentioned *Burkholderia* spp., and 18 of the 20 have orthologs in the phytopathogen *R. solanacearum*. Overall, the order and orientation of these genes are conserved between *R. solanacearum* and the *Burkholderia* spp. described above (Fig. 2). The only differences between the $T3SS2_{bp}$ gene cluster, the $T3SS_{rso}$ gene cluster, and the orthologous clusters in *B. mallei*, *B. thailandensis*, *B. oklahomensis*, and *B. ubonensis*

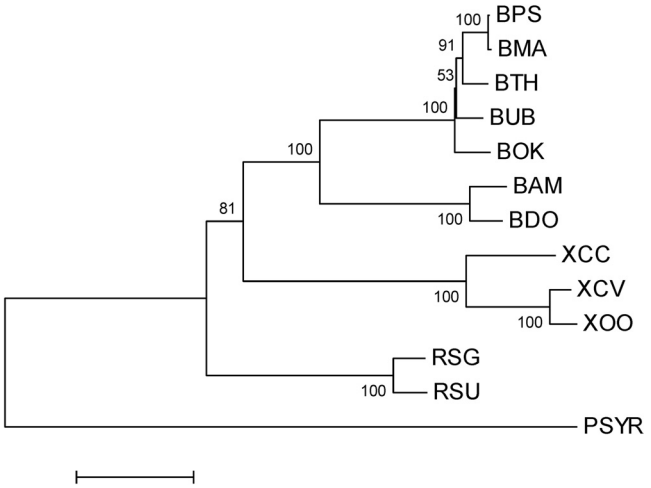

FIG. 1. Phylogenetic tree of 3 conserved T3SS genes from plant pathogens and *Burkholderia* species. Multiple sequence alignments of *hrcV*, *hrcN*, and *hrcC* orthologs from *B. pseudomallei* K96243 (BPS), *B. mallei* ATCC 23344 (BMA), *B. thailandensis* E264 (BTH), *B. ubonensis* Bu (BUB), *B. oklahomensis* C6786 (BOK), *B. ambifaria* AMMD (BAM), *B. dolosa* AU0158 (BDO), *R. solanacearum* UW551 (RSU), *R. solanacearum* GMI1000 (RSG), *X. campestris* pv. campestris ATCC 33913 (XCC), *X. oryzae* pv. oryzae MAFF311018 (XOO), *X. campestris* pv. vesicatoria 85-10 (XCV), and *Pseudomonas syringae* pv. tomato DC3000 (PSYR) were trimmed and concatenated, and a neighbor-joining phylogenetic tree was built using MEGA4 (65). The numbers at nodes are the percent bootstrap support values (1,000 replicates). Scale bar, 10% substitution. All sequences were obtained from IMG.

TABLE 2. Percent amino acid sequence identities of *B. pseudomallei* $T3SS2_{bp}$ proteins with their orthologs in *R. solanacearum* and *X. campestris* pv. vesicatoria

B. pseudomallei protein type and corresponding locus tag	*R. solanacearum* GMI1000		*X. campestris* pv. vesicatoria	
	Protein	% amino acid identity	Protein	% amino acid identity
Hrc protein orthologs				
BPSS1629	HrcT	41	HrcT	40
BPSS1627	HrcN	67	HrcN	72
BPSS1624	HrcJ	60	HrcJ	50
BPSS1621	HrcU	47	HrcU	45
BPSS1620	HrcV	60	HrcV	63
BPSS1618	HrcQ	30	HrcQ	50
BPSS1617	HrcR	62	HrcR	61
BPSS1616	HrcS	57	HrcS	53
BPSS1592	HrcC	44	HrcC	46
Hrp protein orthologs				
BPSS1628	HrpD	33	HrpB7	<10
BPSS1626	HrpF	37	HrcL	32
BPSS1625	HrpH	33	HrpB4	28
BPSS1623	HrpJ	30	HrpB2	29
BPSS1622	HrpK	30	HrpB1	31
BPSS1615	HrpV	26	HpaA	27
BPSS1614	HrpW	34	HrcD	33
BPSS1610	HrpB	38	HrpX	38
Hpa protein orthologs				
BPSS1619	HpaP	36	HpaC	27
BPSS1611	HpaB	53	HpaB	54

are the translocation of $hrpB_{bp}$ to the opposite end of the T3SS cluster and the translocation of the *hrcC* secretin gene to a site 19 kbp upstream. In the remaining *Burkholderia* spp., the location of *hrpB* and the secretin gene are inverted relative to what is found in *B. pseudomallei*, *B. mallei*, *B. thailandensis*, *B. oklahomensis*, and *B. ubonensis*.

The nine core proteins of the T3SS of *R. solanacearum* are encoded by the *hrc* genes. Over half of these proteins average ~60% amino acid identity with their *B. pseudomallei* orthologs (Table 2). The remaining core $T3SS2_{bp}$ proteins have amino acid identities of ~40% with their orthologs in $T3SS_{rso}$, and all are assigned to the same clusters of orthologous groups (COGs) families. A majority of the 10 remaining $T3SS2_{bp}$ proteins have amino acid identities ranging from 33 to 37% with their *R. solanacearum* counterparts. Finally, the predicted transcription units of $T3SS2_{bp}$ (51) are also nearly identical in composition and direction to the experimentally determined ones of $T3SS_{rso}$.

BPSS1612 and BPSS1613 are in a genome location analo-

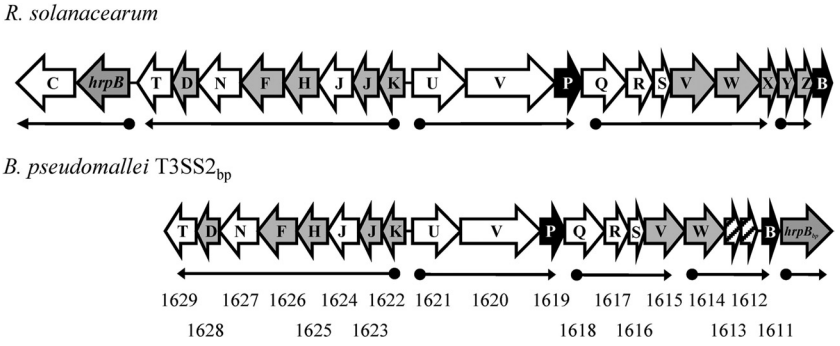

FIG. 2. Organization of the orthologous T3SS gene clusters of *R. solanacearum* and *B. pseudomallei*. The *R. solanacearum* "*hrc*" genes (i.e., the nine core genes conserved in all bacterial T3SS clusters) are denoted by white arrows, the "*hrp*" genes by gray arrows, and the "*hpa*" genes by black arrows. Their orthologs in the *B. pseudomallei* $T3SS2_{bp}$ cluster are similarly colored. The striped arrows indicate genes whose products do not have >25% amino acid sequence identity with any $T3SS_{rso}$ proteins. The determined transcription units for *R. solanacearum*, predicted transcription units for *B. pseudomallei*, and the direction of transcription are denoted by thin black arrows; the filled circles indicate transcription starts. Numbers are the BPSS locus tags for *B. pseudomallei* genes from IMG. The *B. pseudomallei* ortholog of *hrcC* is located 19 kbp upstream from the $T3SS2_{bp}$ cluster (not pictured). The $T3SS2_{bt}$ cluster in *B. thailandensis* (not pictured) is identical to the $T3SS2_{bp}$ cluster.

TABLE 3. *B. pseudomallei* and select *B. thailandensis* genes showing increased transcription in response to $HrpB_{bp}$ overexpression

Locus tag(s)	$p2_{bp}$ box[a]	Description/product(s)[b]	Log_2 relative expression[c]		
			Microarray	qRT-PCR	RNA-Seq
BPSS1611-BPSS1629	+	$T3SS2_{bp}$ cluster	3.5	NA	9.4
BPSS1604-BPSS1609	+	Hypothetical proteins and a two-component regulatory system	3.3	NA	9.3
BPSS1593-BPSS1601	−	Type IV pilus-encoding cluster	2.9	NA	5.5
BPSS0998-BPSS1004	+	Polyketide biosynthesis cluster	1.7	NA	4.8
BPSS1614 (BTH_II0758[d])	+	Ortholog of *R. solanacearum hrpW*	Spot absent	10.6 ± 0.7 (11.5 ± 0.3)	10.6
BPSS1621 (BTH_II0750[d])	+	Ortholog of *R. solanacearum hrcU*	3.4 ± 0.05	10.5 ± 0.1 (10.6 ± 0.6)	9.8
BPSS1623 (BTH_II0748[d])	+	Ortholog of *R. solanacearum hrpJ*	4.4 ± 0.01	12.2 ± 0.1 (12.0 ± 0.5)	11.8
BPSS1592	+	$T3SS2_{bp}$ secretin	4.5 ± 0.1	8.1 ± 0.8	8.2
BPSS1606 (BTH_II0764[d])	+	Hypothetical protein	4.2 ± 0.1	13.2 ± 0.2 (12.8 ± 0.5)	13.1
BPSS1003	+	Hypothetical protein	1.3 ± 0.03	4.7 ± 1.8	4.2
BPSS1004	+	Malonyl CoA-acyl carrier protein transacylase	1.5 ± 0.08	6.3 ± 1.0	8.3
BPSS1323	+	Hypothetical protein	3.9 ± 0.2	13.4 ± 0.4	13.3
BPSS0740	+	Hypothetical protein	3.0 ± 0.3	9.6 ± 0.2	10.5
BPSS0310	+	Putative SET domain protein	3.2 ± 0.2	ND	2.3
BPSS0312	+	Autoinducer-binding transcriptional regulator	2.4 ± 0.4	3.2 ± 0.4	4.3
BPSS1389	+	Putative hemagglutinin/hemolysin	1.9 ± 0.6	6.2 ± 0.5	6.8
BPSS1384	−	Putative membrane protein	4.0 ± 0.1	7.6 ± 1.3	7.8
BPSS0804	−	Hypothetical protein	4.3 ± 0.03	ND	10.0
BPSS1322	−	Transcriptional regulator, AraC family	4.3 ± 0.01	ND	8.6
BPSS0803	−	Hemolysin III-like integral membrane protein	3.3 ± 0.04	ND	6.8
BPSL0006	−	Putative soluble lytic transglycosylase	2.1 ± 0.1	ND	2.8
BPSS1409	−	Hypothetical protein	2.0 ± 0.2	ND	6.4
BPSS0995	−	AraC family transcriptional regulator	1.9 ± 0.1	ND	5.1
BPSS0996	−	Rieske family iron-sulfur cluster-binding protein	2.0 ± 0.3	ND	4.4

[a] +, present; −, absent.
[b] The first 4 genes were manually annotated; the descriptions of the remaining genes are based upon data from IMG (http://img.jgi.doe.gov).
[c] Log_2 ratios (± standard deviations) of gene transcript levels in *B. pseudomallei* or *B. thailandensis* strains overexpressing $HrpB_{bp}$ relative to that of the wild type. The first four rows of data represent the average upregulation of all genes in the indicated cluster. NA, not applicable; ND, not done.
[d] Locus tag of the orthologous gene in *B. thailandensis*.

gous to that of *hrpY* and *hrpX* of $T3SS_{rso}$, which encode the pilin subunit and a pilus assembly protein, respectively (Fig. 2) (68, 69). However, the corresponding amino acid identities are only 18%. Although pilin proteins of plant pathogen T3SSs lack amino acid sequence similarity, predicted amphiphilic helices and coiled-coil secondary structures are usually predicted in most of these proteins, including HrpY (69). BPSS1613 contains a predicted amphiphilic helix and coiled-coil domain (39, 60), indicating that it could be the pilin subunit.

HrpB of *R. solanacearum* ($HrpB_{rso}$), the AraC-type activator that is the master regulator of $T3SS_{rso}$ expression, has 38% amino acid identity with its *B. pseudomallei* ortholog, encoded by BPSS1610 ($HrpB_{bp}$). In addition to the amino acid sequence similarities described above, there are also DNA sequence similarities in the T3SS promoter regions of *B. pseudomallei* and *R. solanacearum* (see below). Taken together, these data suggest that the $T3SS2_{bp}$ genes encode all the proteins needed for the assembly and regulation of a functionally complete plant pathogen-like type III secretion system analogous to the one in *R. solanacearum*.

Transcriptome analysis of *B. pseudomallei* overexpressing $HrpB_{bp}$ reveals its regulon. Because HrpB positively regulates the transcription of the $T3SS_{rso}$ genes and many of its secreted effectors (21), we determined whether $HrpB_{bp}$ activates the expression of $T3SS2_{bp}$ genes in *B. pseudomallei*. Total mRNA from *B. pseudomallei* cells overexpressing $HrpB_{bp}$ was compared to total mRNA from cells with the empty vector by microarray analysis. Overall, 48 genes were upregulated >4-fold when $HrpB_{bp}$ was overexpressed ~10-fold from plasmid pSCR1610a (Table 3). Bulk sequence analysis of cDNA populations derived from one set of the same RNA preparations used as described above with an Illumina Genome Analyzer (RNA-Seq) (76) gave very similar results, except that the magnitude of upregulation in general was >10-fold higher (Table 3). Sixteen of the 20 $T3SS2_{bp}$ genes in all five transcription units were upregulated an average of ~14-fold. Only one of the ~40 genes in the other two T3SSs (BPSS1409 from $T3SS1_{bp}$) appeared to be upregulated. Thus, $HrpB_{bp}$ activates the transcription of the genes encoding $T3SS2_{bp}$ but not the other T3SSs.

Interestingly, 14 of the 17 genes in the 19-kbp region upstream of the $T3SS2_{bp}$ operons (BPSS1593-BPSS1609) were also highly upregulated in response to overexpression of $HrpB_{bp}$ (Table 3). BPSS1593-BPSS1601, which is predicted to be an operon encoding a type IV pilus, was upregulated ~8-fold; this surface structure could be involved in adhesion (50). BPSS1604 and BPSS1605 encode a two-component regulatory system that was upregulated ~8-fold, suggesting that they might comprise downstream components in an $HrpB_{bp}$ regulatory cascade. The type IV pilus operon and two-component regulatory system are also present in the same genome position in all the *Burkholderia* species that have orthologous $T3SS2_{bp}$ gene clusters. Three additional transcriptional regulators were also upregulated in response to $HrpB_{bp}$ overexpression: BPSS0312 (LuxR family) and BPSS1322 and BPSS0995 (both AraC family). These genes were upregulated 9-, 19-, and 4-fold, respectively, and also may be components of an $HrpB_{bp}$ regulatory cascade.

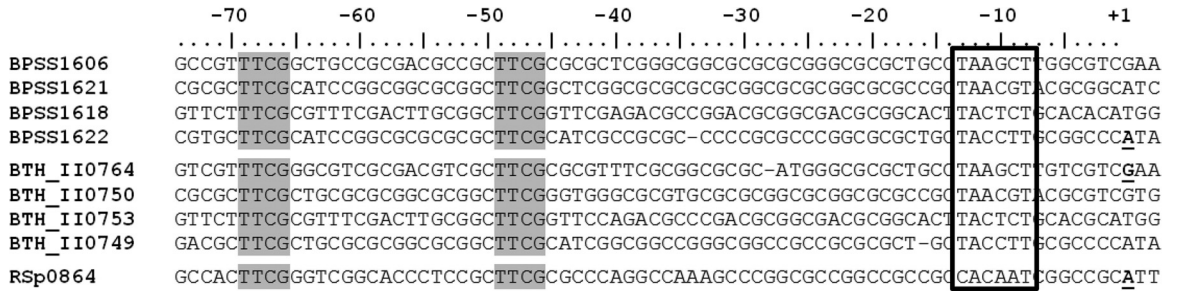

FIG. 3. $p2_{bp}$ boxes in the promoter regions of $HrpB_{bp}$-regulated genes. Predicted promoter regions of $HrpB_{bp}$-regulated genes were aligned manually by using the ATG start codon as an anchor. BPSS promoter regions shown are from genes upregulated >10 fold according to the microarray analysis. *B. thailandensis* (BTH_II) promoter regions shown are from genes orthologous to the *B. pseudomallei* (BPSS) genes. RSp0864 is the promoter region from the HrpB-regulated gene *hrcU* in *R. solanacearum*. Shading indicates the conserved direct repeats, and the open box indicates the predicted −10 region. Transcription start sites are indicated by +1, and those which were experimentally determined are underlined and in bold.

Another notable $HrpB_{bp}$-regulated gene cluster (BPSS0998-BPSS1004) is predicted to include polyketide biosynthesis genes. This cluster was upregulated an average of ~3-fold and is found only in *B. pseudomallei* and *B. mallei*. This gene cluster is similar in organization and composition to a predicted polyketide biosynthesis gene cluster in the distantly related algicidal flavobacterium *Kordia algicida*. BPSS1003 and BPSS1004 from this cluster were upregulated 19- and 313-fold, respectively, in the RNA-Seq analysis.

Several monocistronic genes were also upregulated in response to overexpression of $HrpB_{bp}$. BPSL0006, which is found in all *Burkholderia* spp. harboring $T3SS2_{bp}$, was upregulated 4-fold and encodes a putative soluble lytic transglycosylase whose ortholog in *Xanthomonas oryzae* is predicted to remodel peptidoglycan to allow for assembly of the T3SS apparatus in the cell wall (78). Another gene, BPSS0310, was upregulated 9-fold and encodes a putative SET domain protein, and these are best known for altering eukaryotic gene expression by methylating lysine residues on histones (25). Proteins similar to those encoded by BPSL0006 and BPSS0310 are found in *R. solanacearum* (with >30% amino acid identity), but neither is regulated by $HrpB_{rso}$ (48). Finally, some of the genes most strongly activated by $HrpB_{bp}$ (>8-fold), BPSS1323, BPSS1609, BPSS0740, and BPSS1384, encode hypothetical proteins without PFAM domains or COG assignments. Interestingly, all of these encoded hypothetical proteins, as well as that encoded by BPSS0310, are predicted to be T3SS-secreted effectors by the Effective T3 prediction tool (http://www.effectors.org/index.jsp), based upon their 50 N-terminal amino acids (4).

To verify and more accurately quantify upregulation measurements from the microarray analysis, mRNA levels of select genes were measured by qRT-PCR. Four $T3SS2_{bp}$ genes from separate predicted transcription units (BPSS1623, BPSS1621, BPSS1614, and BPSS1592) each showed an expression increase of >250-fold when $HrpB_{bp}$ was overexpressed (Table 3). As a control, the expression levels of BPSS1180, a non-$T3SS2_{bp}$ gene which was not upregulated in the microarray, was measured by qRT-PCR and showed no increase in expression. We also verified the $HrpB_{bp}$-dependent transcription activation of 8 non-$T3SS2_{bp}$ genes. Four showed expression increases of >190-fold, two others were upregulated ~75-fold, and the remainder were upregulated ~15-fold. These values are >10-fold higher than the values obtained from the microarray analysis. However, this is not unexpected, as microarray analysis has been shown to underestimate the extent of mRNA expression change (77).

$HrpB_{bp}$ regulates transcription of $T3SS2_{bt}$ and $T3SS_{rso}$. Given the high (>90%) nucleotide sequence identity and synteny between the $T3SS2_{bp}$ cluster in *B. pseudomallei* and the T3SS cluster in *B. thailandensis* ($T3SS2_{bt}$), we overexpressed $HrpB_{bp}$ in *B. thailandensis* and used qRT-PCR to measure $HrpB_{bp}$-dependent upregulation of the orthologs of the $T3SS2_{bp}$ genes from *B. pseudomallei* that were analyzed (BTH_II0748, BTH_II0750, and BTH_II0758). We also measured the expression change of a gene adjacent to the $T3SS2_{bt}$ cluster (BTH_II0764), whose ortholog in *B. pseudomallei* (BPSS1606) was the most highly upregulated gene. All of these genes were upregulated >1,000-fold (Table 3), suggesting that $HrpB_{bp}$ also strongly regulates $T3SS2_{bt}$ in *B. thailandensis*.

These results and the similarities between $T3SS2_{bp}$ regulation and $T3SS_{rso}$ regulation prompted the analysis of the ability of $HrpB_{bp}$ to activate transcription of T3SS genes in the heterologous host *R. solanacearum*. The mRNA levels of three $T3SS_{rso}$ genes in *R. solanacearum* overexpressing $HrpB_{bp}$ were measured by qRT-PCR. These genes, *hrpK*, *hrpW*, and *hrcV*, are in the same transcription units as the $T3SS2_{bp}$ genes tested in *B. pseudomallei* and *B. thailandensis*. Transcription of these genes increased ~4.5-fold when $HrpB_{bp}$ was overexpressed. Although $HrpB_{bp}$ shares only 38% amino acid identity with HrpB from *R. solanacearum*, it can still activate the expression of $T3SS_{rso}$ genes, albeit much less than in *B. pseudomallei*.

Several $HrpB_{bp}$-regulated promoters have a conserved DNA motif. In *R. solanacearum*, HrpB positively regulates transcription of the genes encoding the T3SS and its secreted effectors via a conserved DNA sequence called the hrp_{II} box located 46 bp upstream from the transcription start site (21). Manual alignment of the promoter region sequences of $HrpB_{bp}$-regulated genes in *B. pseudomallei* and *B. thailandensis* revealed the presence of a motif identical to the hrp_{II} box (Fig. 3). This motif (designated the $p2_{bp}$ box) consists of two 4-bp direct repeats separated by 16 bp (TTCG-N_{16}-TTCG) and is located ~32 bp upstream from a putative −10 region.

We employed 5′ RACE to determine the transcription start sites of two highly $HrpB_{bp}$-regulated genes identified by both

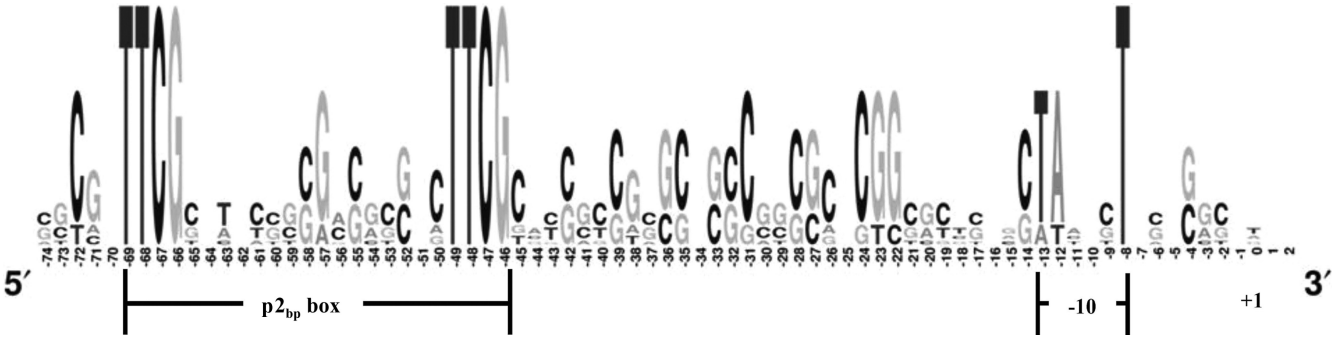

FIG. 4. Sequence conservation in the promoter regions of HrpB$_{bp}$-regulated genes. The figure was generated by using a manual alignment of the promoter regions of seven *B. pseudomallei* genes upregulated >200-fold when HrpB$_{bp}$ is overexpressed and Weblogo (20). The locations of the p2$_{bp}$ box, −10 region, and transcription start site (+1) are indicated.

microarray and qRT-PCR analysis, BPSS1622, a *B. pseudomallei* T3SS2$_{bp}$ gene, and BTH_II0764, a *B. thailandensis* gene encoding a hypothetical protein adjacent to the two-component regulatory system that is also highly regulated. For both genes, the transcription start site was mapped to a position 45 bp downstream from the 3′ end of the p2$_{bp}$ box (Fig. 3). This confirmed the location of the predicted −10 region, which is nearly identical to that found in hrp$_{II}$ box promoters in *R. solanacearum* (21).

Several HrpB$_{bp}$-regulated genes not associated with T3SS2$_{bp}$ also possess a p2$_{bp}$ box in their predicted promoter regions. Using Weblogo (20), we analyzed the nucleotide sequence of promoter regions of the seven most highly HrpB$_{bp}$-regulated genes determined by qRT-PCR (Fig. 4). This analysis identified most, if not all, of the conserved nucleotides in these HrpB$_{bp}$-regulated promoters. In addition to the direct repeats and the −10 region, other conserved positions around position −72, between positions −58 and −55, and between positions −22 and −39, especially positions −22 to −31, were identified. These additional regions of sequence conservation may contribute to promoter strength because they are not conserved in weakly upregulated genes with a p2$_{bp}$ box (data not shown). One feature that is notably absent from these promoters is a −35 consensus sequence. However, this feature is also absent in the hrp$_{II}$ box promoters of *R. solanacearum* (21).

The p2$_{bp}$ box is essential for transcription activation by HrpB$_{bp}$. The data described above imply that the p2$_{bp}$ box is homologous to the hrp$_{II}$ box and likely functions with HrpB$_{bp}$ to activate transcription in a manner similar to HrpB-mediated transcription activation in *R. solanacearum*. To confirm this, several *lacZ* reporter fusions that either have or lack the p2$_{bp}$ box were constructed and transferred into wild-type, HrpB$_{bt}$ (HrpB$_{bp}$ ortholog) mutant, or HrpB$_{bp}$-overexpressing HrpB$_{bt}$ mutant *B. thailandensis* strains and assayed for HrpB$_{bp}$-dependent promoter activity by using β-galactosidase activity. *B. thailandensis* was used because we could not find or readily construct a fusion vector approved for use in *B. pseudomallei* and because *B. pseudomallei* and *B. thailandensis* are so closely related (>90% DNA sequence identity). Relative to the wild type, the HrpB$_{bp}$-dependent promoter activities of pPR1606 and pPR0764 (Table 1), which contain promoter fragments from orthologous *B. pseudomallei* and *B. thailandensis* genes (BPSS1606 and BTH_II0764, respectively), decreased ~5-fold when HrpB$_{bt}$ was inactivated but increased ~83-fold in the HrpB$_{bp}$-overexpressing strain, suggesting HrpB$_{bp}$-mediated transcription activation. The promoter region of BTH_II0764 is predicted to have a divergent promoter with two p2$_{bp}$ boxes, oriented in opposite directions. p2$_{bp}$ box 1 is likely associated with the divergently transcribed upstream gene, BTH_II0763, and deleting it (pPR0764Md) produced no significant change in HrpB$_{bp}$-dependent promoter activity (Fig. 5). However, deleting p2$_{bp}$ box 2 (pPR0764Tr) showed dramatically reduced promoter activity, demonstrating that p2$_{bp}$ box 2 is required for HrpB$_{bp}$-dependent transcription activation of BTH_II0764. Furthermore, the reciprocal recognition of

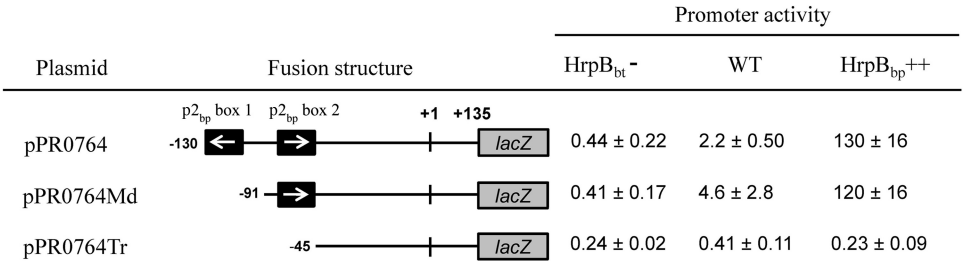

FIG. 5. Analysis of p2$_{bp}$ box requirement for transcription activation by HrpB$_{bp}$. Mutants with 5′ deletions of the promoter region of BTH_II0764 were cloned into a *lacZ* reporter plasmid and transferred into wild-type (WT), HrpB$_{bt}$-deficient (HrpB$_{bt}$⁻), and HrpB$_{bt}$-deficient but HrpB$_{bp}$-overexpressing (HrpB$_{bp}$++) *B. thailandensis* strains. Promoter activity was monitored by β-galactosidase assay, and activities are reported in Miller units/100. Numbers indicate nucleotide positions relative to the transcription start; white arrows indicate the orientation of the p2$_{bp}$ box. +1, transcription start site; +135, first nucleotide of *lacZ* CDS.

TABLE 4. Effect of nucleotide substitutions in $p2_{bp}$ box direct repeats on $HrpB_{bp}$-dependent promoter activity

Plasmid	$p2_{bp}$ box sequence[a]	Promoter activity[b]	
		WT	$HrpB_{bp}$++
	−70 −60 −50		
pPR0764	GT**TTCG**GGCGTCGCGACGTCGC**TTCG**CG	2.2 ± 0.49	140 ± 17
pPR0764A2	GT**AACG**GGCGTCGCGACGTCGC**TTCG**CG	1.3 ± 0.70	4.0 ± 1.3
pPR0764B2	GT**TTCG**GGCGTCGCGACGTCGC**AACG**CG	1.1 ± 0.60	5.6 ± 2.5
pPR0764C2	GT**AACG**GGCGTCGCGACGTCGC**AACG**CG	0.56 ± 0.05	0.52 ± 0.24
pPR07641a	GT**TTAG**GGCGTCGCGACGTCGC**TTCG**CG	0.43 ± 0.07	0.42 ± 0.05
pPR07648a	GT**TTCG**TGCATCGCGACGTCTC**TTCG**CG	Not done	145 ± 38

[a] The bold, underlined nucleotides are the direct repeats of the $p2_{bp}$ box; shadowed nucleotides indicate substitutions. Nucleotide positions relative to transcription start are given above.

[b] β-Galactosidase activity directed by each plasmid in wild-type (WT) and $HrpB_{bp}$-overexpressing ($HrpB_{bp}$++) *B. thailandensis* strains in Miller units/100.

B. pseudomallei and *B. thailandensis* $p2_{bp}$ box promoter fragments by $HrpB_{bp}$ and $HrpB_{bt}$ indicates homology of these transcriptional regulators.

For *R. solanacearum*, growth in minimal medium with glutamate increases the expression of HrpB-regulated genes relative to growth in rich media. To determine if this is also the case for *B. thailandensis*, we assayed the $HrpB_{bt}$-dependent promoter activities of wild-type *B. thailandensis* (pPR0764) grown in LB and M9–20 mM glutamate. There was no difference in $HrpB_{bt}$-regulated promoter activity in cells from these two types of media (data not shown), suggesting that $HrpB_{bt}$ expression, as well as subsequent induction of $HrpB_{bt}$-regulated genes in *B. thailandensis*, is regulated by different environmental signals than the mechanism in *R. solanacearum*.

Determination of nucleotides involved in $HrpB_{bp}$-dependent transcription activation. To assess the functional importance of specific nucleotides in the direct repeats of the $p2_{bp}$ box, we mutated the $p2_{bp}$ box in plasmid pPR0764 by using site-directed mutagenesis or error-prone PCR and measured promoter activities in various *B. thailandensis* strains (Table 4). In the $HrpB_{bp}$-overexpressing strain, when TT in either the first or second repeat of the $p2_{bp}$ box (positions −68 and −69 or −48 and −49) was changed to AA, the promoter activity decreased ~30-fold. When TT was changed to AA in both repeats, $HrpB_{bp}$ activation of the promoter was lost. Changing the third base in the first repeat, from C to A at position −67, also abolished the ability of the promoter to be activated by $HrpB_{bp}$. A mutant harboring three mutations in the spacer between the direct repeats of the $p2_{bp}$ box retained $HrpB_{bp}$-dependent activity. This suggests that specific bases at −49, −48, and −67 to −69 are crucial for $HrpB_{bp}$-dependent transcription activation from this promoter but that the sequence of the spacer between the repeats is not critical for promoter activity.

***B. thailandensis* fails to demonstrate a phytopathogenic phenotype.** Given the remarkable similarity of $T3SS2_{bp}$ to the T3SSs required for virulence of several plant pathogens, it is plausible that $T3SS2_{bp}$ in *Burkholderia* spp. is also involved in plant pathogenesis. Lee et al. (34) found that soaking tomato plantlets in suspensions of *B. thailandensis* E264 or *B. pseudomallei* for 7 days produced disease-like symptoms (i.e., leaf vein blackening, wilting, and necrosis) and that development of these symptoms was delayed when using a $T3SS2_{bp}$ mutant. We treated tomato plantlets identically with the same *B. thailandensis* E264 strain but observed only a wilting symptom. However, soaking plantlets in suspensions of nonphytopathogenic *P. putida* ATCC 12633 or media alone also caused wilting. Moreover, we found that after 36 h, populations of all strains in the soaking solution exceeded 10^8 cells ml^{-1}. When we petiole inoculated ~10^6 cells of *B. thailandensis* E264, *B. thailandensis* DW503, or *P. putida* into larger, more mature tomato plants, they remained asymptomatic for 7 days. This is in stark contrast to the plants inoculated with ~10^4 cells of *R. solanacearum*, which showed extensive wilting after only 3 days. These wilted plants harbored >10^9 cells g^{-1} of stem, while populations of *B. thailandensis* strains in asymptomatic plants were only 10^4 cells g^{-1}. We tested a second plant host (*Arabidopsis thaliana*) by infiltrating leaves with 10^7 or 10^8 cells ml^{-1} of the *B. thailandensis* strains used as described above. After 21 days, these plants were asymptomatic. Consistent with previous reports (10), plants infiltrated with the same amounts of *R. solanacearum* were chlorotic and wilted by day 12. Symptomatic *A. thaliana* plants harbored 10-fold more cells than asymptomatic plants.

A hallmark of nearly all plant-pathogenic bacteria is the ability to produce a hypersensitive response in tobacco (13, 23, 79). Tobacco leaves infiltrated with 10^9 cells ml^{-1} of *B. thailandensis* or *P. putida* did not elicit a hypersensitive response after 7 days, while those infiltrated with the same amount of *R. solanacearum* developed a hypersensitive response after 2 days. Those leaves infiltrated with 10^7 or 10^8 cells ml^{-1} of *R. solanacearum* showed a hypersensitive response by day 4. Taken together, these results suggest that *B. thailandensis* is not an aggressive or typical plant pathogen.

DISCUSSION

We investigated the organization and regulation of the $T3SS2_{bp}$ gene cluster, one of the three T3SS gene clusters in *B. pseudomallei*. Orthologous gene clusters containing this T3SS cluster are found in *B. mallei*, *B. thailandensis*, *B. oklahomensis*, *B. ubonensis*, *B. ambifaria*, and *B. dolosa* but not in any of the 12 other sequenced *Burkholderia* species. Although our analyses clearly showed that this T3SS is very closely related in gene content, organization, and sequence to one required by the plant pathogens *R. solanacearum* ($T3SS_{rso}$) and *Xanthomonas* spp. for causing disease, the role of $T3SS2_{bp}$ in

plant pathogenesis or any other biological process involving any of the *Burkholderia* spp. harboring it remains unclear.

In addition to finding genetic similarities between the $T3SS2_{bp}$ and $T3SS_{rso}$ gene clusters, we also found remarkable regulation similarities. In *R. solanacearum*, transcription activation of >20 operons and genes associated with $T3SS_{rso}$ is directly mediated by the AraC-type regulator HrpB via interaction with the conserved hrp_{II} box motif (21). We identified an identical motif in *B. pseudomallei* ($p2_{bp}$ box) that is similarly required for $HrpB_{bp}$-dependent transcription activation of $T3SS2_{bp}$ genes, and we showed that, just as hrp_{II} box mutations negatively affect transcription activation by HrpB in *R. solanacearum* (21), $p2_{bp}$ box mutations eliminate $HrpB_{bp}$-dependent transcription activation. The PIP box (TTCGC-N_{15}-TTCGC) in *Xanthomonas* spp. is nearly identical to the hrp_{II} and $p2_{bp}$ boxes and is likewise required for HrpX (the ortholog of HrpB and $HrpB_{bp}$)-mediated activation of T3SS transcription (66). HrpB from *R. solanacearum* can partially complement a HrpX mutant in *X. campestris* pv. vesicatoria (73), and we showed that $HrpB_{bp}$ from *B. pseudomallei* can activate $T3SS_{rso}$ gene expression. Thus, HrpB and the sequences it uses to regulate transcription are highly conserved across at least 3 genera. Additionally, *Acidovorax avenae* subsp. *citrulli* AAC00-1, another plant pathogen in the *Betaproteobacteria*, also has an HrpB ortholog and PIP boxes upstream from some of its T3SS genes. However, HrpB regulation in *A. avenae* subsp. *citrulli* has not been explored.

The expression of *hrpB* in *R. solanacearum* is induced either through a plant cell contact-dependent regulatory cascade or by an unidentified signal that can be mimicked by growth in minimal media. The *B. pseudomallei* and *B. thailandensis* genomes do not contain orthologs corresponding to any of the regulatory proteins involved in the plant cell contact-dependent pathway, and contrary to what was found for *R. solanacearum* (3), growth in minimal medium containing glutamate did not induce expression of $hrpB_{bt}$ in *B. thailandensis*. This suggests that the signal inducing $hrpB_{bt}$ expression in *B. thailandensis*, and by extension $hrpB_{bp}$ expression in *B. pseudomallei*, is different from the inducing signal in *R. solanacearum*.

In *R. solanacearum*, HrpB is the terminal regulatory protein for the activation of T3SS transcription. Transcriptome analysis of *R. solanacearum* overexpressing HrpB showed that it does not positively regulate any other regulators (48). However, HpaR, a transcriptional regulator that is positively regulated by HrpX in *Xanthomonas campestris* pv. campestris, is required for pathogenicity but does not regulate any T3SS genes (72). While there is no ortholog of HpaR in *B. pseudomallei*, our analysis did indicate that a two-component regulatory system, a LuxR family regulator, and two AraC-type regulators may be downstream components of a $HrpB_{bp}$ regulatory cascade. The multiple proteins that may be involved in the $HrpB_{bp}$ regulatory cascade underscore the greater complexity of the $HrpB_{bp}$ regulon and warrant further study.

HrpB in *R. solanacearum* positively regulates not only the expression of $T3SS_{rso}$ but also the expression of many effectors secreted through $T3SS_{rso}$ (22, 45). Transcriptome analyses, genetic screens, and bioinformatic searches for hrp_{II} boxes has identified 72 HrpB-regulated effectors in *R. solanacearum* (44, 45, 48). In contrast, the transcriptome analysis we performed identified only 33 $HrpB_{bp}$-upregulated genes in addition to the $T3SS2_{bp}$ cluster. Of these genes, BPSS0310, BPSS0740, BPSS1609, BPSS1323, and BPSS1384 all possess $p2_{bp}$ boxes in their predicted promoter regions, and the proteins they encode are predicted to be T3SS secreted. BPSS0310 is particularly interesting because it encodes a putative SET domain protein; some members of this family are histone lysine methyltransferases involved in modulating eukaryotic gene expression (25). It is tempting to speculate that this protein is a *bona fide* effector that could affect host gene expression after it is injected by $T3SS2_{bp}$. None of the other predicted T3SS-secreted proteins show amino acid sequence identity to any known plant or animal pathogen T3SS effectors, or any other proteins, and thus may represent novel effectors.

Two notable gene clusters in *B. pseudomallei* that were also $HrpB_{bp}$ regulated are a type IV pilus (Tfp) gene cluster located 8 kbp away from $T3SS2_{bp}$ and a predicted polyketide biosynthesis cluster located ~86 kbp away from $T3SS2_{bp}$. This Tfp system is orthologous to the Tfp that contributes to virulence of *Yersinia pseudotuberculosis* in mice (18), where it was suggested to play a role in colonization of intestinal mucosa. The Tfp in *B. pseudomallei* could be involved in adhesion to host cells to facilitate injection of effectors by $T3SS2_{bp}$.

The $HrpB_{bp}$-regulated polyketide gene cluster may be involved in the synthesis of a type III polyketide, since it contains BPSS1002, encoding a predicted type III polyketide synthase with the Cys-His-Asn catalytic triad required for the condensation reactions (5). These proteins are in the chalcone synthase superfamily and generate aromatic polyketides by using various starter and extender substrates (i.e., long-chain fatty acyl coenzyme A [acyl-CoA] thioester starter and malonyl-CoA extenders) (5, 47). It is possible that BPSS1002 and its adjacent genes produce a toxin or antibiotic that may function independently or as an enhancer of $T3SS2_{bp}$ function.

Although *B. thailandensis* and *B. pseudomallei* were reported to cause disease-like symptoms on tomato plantlets (34), our analysis indicates that *B. thailandensis* does not produce symptoms characteristic of a plant pathogen on tomato, tobacco, or *A. thaliana* plants. Moreover, an attempt to demonstrate phytopathogenicity of *B. thailandensis* on *Oryza sativa* (rice) plants was similarly unsuccessful (34). These results, coupled with observation that, unlike those of *R. solanacearum* and *Xanthomonas* spp., the genomes of *B. thailandensis* and *B. pseudomallei* encode no plant cell wall-degrading enzymes (e.g., pectate lyases, polygalacturonases, or endoglucanases) (37, 70), call into question any suggestion that *B. thailandensis* and, by extension, *B. pseudomallei* are typical plant pathogens. In fact, of the >30 described *Burkholderia* spp., only 6 are reported to cause plant disease (17, 40), and none of these has a T3SS orthologous to $T3SS2_{bp}$.

If *B. pseudomallei* is not a plant pathogen, then what could be the function of $T3SS2_{bp}$? Shotgun proteomic analysis has shown that at least some $HrpB_{bp}$-activated genes (encoding the secretins of Tfp and $T3SS2_{bp}$) are not just transcribed pseudogenes or relics but are translated into protein and properly localized in the outer membrane only when $HrpB_{bp}$ is overexpressed in *B. pseudomallei* (unpublished data). A possible $T3SS2_{bp}$ function could be a role in pathogenesis or manipulation of other organisms, such as fungi, amoebae, or algae. Interestingly, treatment of pea seeds with *B. ambifaria* AMMD, which contains a T3SS orthologous to $T3SS2_{bp}$, con-

trols seed rot and damping-off caused by *Pythium* spp. and in some cases controls root rot caused by *Aphanomyces euteiches* (16, 33). Although it has been reported that *B. pseudomallei* can invade the spores and attach to the hyphae of the arbuscular mycorrhizal fungus *Gigaspora decipiens* (35) and that *B. pseudomallei* and *B. thailandensis* can survive inside the amoeba *Dictyostelium discoideum* (28a), the role of $T3SS2_{bp}$ in its interaction with these organisms remains to be investigated.

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