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A Type IV Pilin, PilA, Contributes to Adherence of *Burkholderia pseudomallei* and Virulence In Vivo

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The *Burkholderia pseudomallei* K96243 genome contains multiple type IV pilin-associated loci, including one encoding a putative pilus structural protein (*pilA*). A *pilA* deletion mutant has reduced adherence to human epithelial cells and is less virulent in the nematode model of virulence and the murine model of melioidosis, suggesting a role for type IV pili in *B. pseudomallei* virulence.

Burkholderia pseudomallei is the causative agent of melioidosis, a disease endemic to southeast Asia and northern Australia (9, 21, 42). Infections occur via inhalation or percutaneous inoculation, and clinical manifestations include subclinical infections, acute septicemia, and chronic disease (42). *B. pseudomallei* can infect almost any host organ, is resistant to many antibiotics, and can persist for long periods (6). Both *B. pseudomallei* and its close relative *Burkholderia mallei* are potential bioterrorist agents listed by the Centers for Disease Control and Prevention (17, 42).

A number of factors are associated with *B. pseudomallei* virulence, including products secreted by the general secretory pathway, type III secretory systems, flagella, lipopolysaccharide, and capsule (1, 7, 11–13, 29, 30, 35, 36). Despite these studies, little is known about how *B. pseudomallei* causes disease.

Adherence is an important virulence mechanism mediated by carbohydrate molecules, pilus, and nonpilus adhesins (14, 16, 20, 34, 37). Type IV pili (TFP) are important for virulence in many gram-negative bacteria and are divided into two subclasses, IVA and IVB, based on the presence of conserved motifs (38). The Flp subgroup of type IVB pili are shorter than other pilins and have a characteristic Flp motif (18).

B. pseudomallei adheres to human epithelial cell lines, but the molecular basis for this adherence is unknown (5). We describe the identification of multiple TFP-associated loci in *B. pseudomallei* and show that a TFP gene homologue is required for efficient adherence of *B. pseudomallei* to cultured cells and for virulence in vivo.

***B. pseudomallei* TFP genes.** We identified eight TFP-associated loci (designated TFP1 to TFP8) in the *B. pseudomallei* K96243 genome (http://www.sanger.ac.uk/Projects/B_pseudomallei/) by in silico probing with multiple pilin homologues and biogenesis proteins; five such loci contain one or more type IV pilin subunits (Table 1). The presence of two type IVB subunits in TFP7 and TFP8 suggests that *B. pseudomallei* K96243 may synthesize pili with a composite architecture (41, 43).

Analysis of TFP1 and PilA. *B. pseudomallei* K96243 open reading frame (ORF) BPSL0782 was designated *pilA* because the full-length predicted gene product shares 49% similarity (32% identity) to PilA from *Pseudomonas aeruginosa* (GenBank accession no. AAL12242). This locus was designated TFP1 (Table 1). While ORFs downstream of *pilA* in *P. aeruginosa* are clearly pilus associated (reviewed in reference 10), ORFs flanking *pilA* in *B. pseudomallei* K96243 differ substantially and may not be involved with pilin biogenesis.

pilA is predicted to encode the only type IVA pilin in *B. pseudomallei* K96243; it possesses the conserved glycine and phenylalanine residues of type IVA pilins, between which is the predicted leader sequence cleavage site, and also the invariant glutamic acid residue 5 amino acids from the mature N terminus, associated with most pilin types (Fig. 1) (38). The PilA leader is predicted to be 40 amino acids long, based on an upstream Shine-Dalgarno sequence; this sets it apart from other IVA pilins, which have shorter leader sequences (Fig. 1). Alternative *pilA* start codons are not associated with Shine-Dalgarno sequences. *Ralstonia solanacearum*, also from the family *Burkholderiaceae*, has a type IVA pilin with a longer leader sequence (15 amino acid residues) (19).

A number of consecutive arginine residues occur in the putative signal sequence, reminiscent of a twin-arginine translocation secretion signal; however, a consensus motif (26) is not evident, or the arginine residues are too close to the putative cleavage site.

PCR with *pilA*-flanking primers amplified the gene in *B. pseudomallei* strains of diverse origin (Table 2 shows the strains studied). The predicted amino acid sequence was conserved in all strains where sequencing was undertaken (results not shown).

Construction of an unmarked *pilA* deletion mutant strain, JAB16. An allelic-exchange mutant (JAB16) was generated which contains an unmarked in-frame 546-bp deletion in the

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TABLE 1. Summary of TFP loci identified in the *B. pseudomallei* K96243 genome

Locus	Gene	Amino acids[a]	Subunit/other homologue (organism; GenBank accession no.)[b]	% Similarity/% identity (amino acids[c])
Chromosome 1				
TFP1	BPSL0782	207	Type IVA pilus subunit PilA (*Pseudomonas aeruginosa*; AAL12242)	49/32 (217)
TFP2	BPSL1821	63	Type IVB pilus subunit Flp1 (*Actinobacillus actinomycetemcomitans*; AAN75204)	42/24 (76)
TFP3	BPSL1899	56	Type IVB pilus subunit Flp1 (*Actinobacillus actinomycetemcomitans*; AAN75204)	46/28 (75)
TFP4	BPSL2752[d]	150	Type IV prepilin leader protein PilE (*Pseudomonas aeruginosa*; AAA79363)	46/26 (157)
	BPSL2756[d]	186	Type IV prepilin leader protein FimT (*Pseudomonas aeruginosa*; AAB39270)	38/24 (189)
TFP5	BPSL3008[e]	419	Type IV pilus biogenesis protein PilB (*Pseudomonas aeruginosa*; A35384)	47/34 (569)
TFP6	BPSL3170[e]	442	Type IV pilus biogenesis protein PilQ (*Pseudomonas aeruginosa*; S37345)	36/22 (714)
Chromosome 2				
TFP7	BPSS1593	557	Type IVB minor pilus subunit PilV (*Escherichia coli*; AAL05526)	33/21 (561)
	BPSS1595	184	Type IVB major pilus subunit PilS (*Escherichia coli*; BAA77979)	43/24 (206)
TFP8	BPSS2185	56	Type IVB pilus subunit Flp1 (*Actinobacillus actinomycetemcomitans*; AAN75204)	43/22 (75)
	BPSS2186	72	Type IVB pilus subunit Flp1 (*Actinobacillus actinomycetemcomitans*; AAN75204)	48/28 (82)

[a] Number of amino acids in the predicted protein.
[b] Homologues were assigned based on TBLASTN (24) searches in Artemis release 4 (31). Homologues reported are those that have been functionally characterized and published.
[c] Number of residues including gaps in the full-length protein alignment.
[d] Predicted peptides contain prepilin-like leader sequences.
[e] ORF corresponds to the first gene in the predicted locus, as no subunit is present.

pilA gene (32). Briefly, the deleted *pilA* allele was constructed by PCR and transferred to the suicide vector pDM4 to give pAEH16. This was conjugated from JABEC16 to *B. pseudomallei* K96243, and merodiploid integrants were selected. One such colony, JAB16.1x, was cultured without selection and plated onto medium lacking sodium chloride but containing 15% sucrose to enrich for excision of integrated vector DNA (2), resulting in either a wild-type or deleted *pilA* allele. Chloramphenicol sensitivity (Cm^s) was assessed, and Cm^s colonies were analyzed by PCR (data not shown) and Southern blotting (Fig. 2) to distinguish *pilA* mutants from wild type. Three of the first eight Cm^s colonies screened contained the deleted *pilA* allele, and one was designated JAB16.

JAB16 has reduced adherence to human epithelial cell lines. The interaction of *B. pseudomallei* K96243 or JAB16 with human respiratory cell lines was studied. Cell lines were cultured and prepared as previously described (40). Bacterial inocula were prepared from overnight cultures grown in nutrient broth, incubated statically for 16 h at 37°C. Monolayers were infected with diluted bacterial cultures ($\sim 10^5$ CFU/ml) for 1 h at 37°C, and inocula were enumerated by plate counts. Nonadherent bacteria were removed by five washes with phosphate-buffered saline. Monolayers were lysed with 0.1% (vol/vol) Triton X-100 for 30 min at 37°C, and adherent–cell-associated bacteria were enumerated by plate counts. JAB16 adhered significantly less than the K96243 parent to A549, BEAS-2B, and RPMI-2650 cell lines ($P < 0.01$) (Fig. 3A). These data suggest a role for *pilA* in the adherence of *B. pseudomallei* in vitro.

JAB16 exhibits reduced virulence in nematode worms. The soil nematode *Caenorhabditis elegans* is susceptible to *B. pseudomallei* (15). *C. elegans* strain N2 nematodes which had been synchronized to the L4 stage (39) and suspended in K medium (33) were exposed to a lawn of JAB16 or K96243 at 25°C on nematode growth medium agar, and nematode survival was recorded. *C. elegans* started to die by 32 h postinfection, regardless of the infecting strain, but worms infected with JAB16 survived significantly longer than K96243-infected worms (Fig. 3B), 99% of which were dead by 58 and 40 h, respectively ($P < 0.001$). A *P. aeruginosa pilA* mutant displayed reduced piliation and was also deficient for secretion of proteins by the general secretory pathway (23). While this could be the case with JAB16, O'Quinn et al. showed that a *B. pseudomallei* general secretory pathway mutant, deficient in secretion, was unable to delay the time to death of *C. elegans* (27). Since JAB16 adheres less in cell culture, we suggest that the mechanism by which JAB16 delays the time to death of nematodes is due to a reduced-adherence phenotype.

JAB16 is attenuated in a murine model of infection. Groups of six BALB/c mice were challenged with different doses of

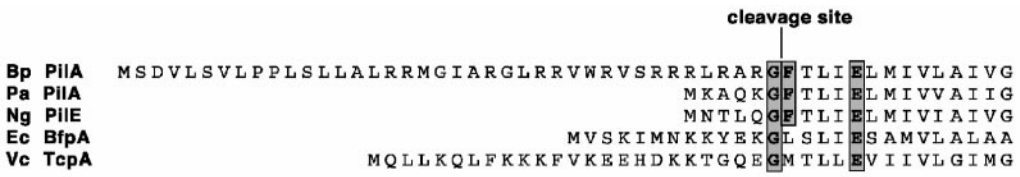

FIG. 1. Comparison of the N-terminal amino acid sequence of *B. pseudomallei* K96243 PilA with representative pilins from the type IVA and IVB subclasses. Species abbreviations: Bp, *B. pseudomallei* strain K96243 PilA (IVA); Pa, *P. aeruginosa* strain PAK PilA (IVA; P02973); Ng, *Neisseria gonorrhoeae* strain MS11 PilE (IVA; CAA47307); Ec, *Escherichia coli* strain O127:H6 BfpA (IVB; P33553); Vc, *Vibrio cholerae* strain classical O1 Z17561 TcpA (IVB; CAA45455). Conserved glycine (G) and phenylalanine (F) residues (F is conserved in type IVA pilins) are highlighted, between which the signal peptides are predicted to be cleaved. The invariant glutamic acid (E) residue associated with most pilins is also indicated.

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
B. pseudomallei strains investigated for conservation of *pilA* by PCR and sequencing[a]		
576 (AY598724)	Clinical isolate, Thailand	PHL[b]
2889 (AY598726)	Pneumonic clinical isolate, Thailand	PHL[b]
NCTC4845 (AY598725)	Monkey isolate	NCTC[c]
B. pseudomallei strains investigated for conservation of *pilA* by PCR		
08	Clinical isolate	4
GCH	Clinical isolate	Gold Coast Hospital[d]
RBH	Clinical isolate	4
06	Environmental isolate	4
GF1	Environmental isolate	4
GF2	Environmental isolate	4
THP375	Environmental isolate	4
B. pseudomallei		
K96243	Clinical isolate, Gen^r Str^r Chl^s, Thailand	Siriraj Hospital[e]
JAB16.1x	K96243 derivative; merodiploid strain; Δ*pilA* (nucleotides 67–612)::pAEH16 (*cat sacBR oriT oriR6K*); Gen^r Str^r Chl^r	This work
JAB16	K96243 derivative; unmarked deletion mutant strain; Δ*pilA* (nucleotides 67–612); Gen^r Str^r Chl^s	This work
E. coli		
OP50	Uracil auxotroph	3
S17.1 (λ*pir*)	RP4-2-Tc::Mu Km::Tn*7* Tp Sm (λ*pir*) *phoA20 thi-1 rpsE rpoB*	28
JABEC16	S17.1 (λ*pir*) containing pAEH16	This work
Plasmids		
pDM4	Suicide vector, *sacBR oriT oriR6K* Chl^r	25
pAEH16	pDM4 containing the *pilA* deletion construct	This work

[a] Numbers in parentheses are GenBank accession numbers.
[b] Supplied by Ty Pitt, Central Public Health Laboratory, Colindale, United Kingdom.
[c] National Collection of Type Cultures.
[d] Supplied by D. Alfredson, Gold Coast Hospital, Gold Coast, Australia.
[e] Supplied by S. Songsivilai, Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

JAB16 and K96243, by either the intraperitoneal route or the intranasal route (22). JAB16 was not attenuated compared to K96243 via the intraperitoneal challenge route (data not shown). In contrast, JAB16 was less virulent than K96243 via the intranasal route but only at low challenge doses ($P < 0.05$) (Fig. 3C). This may be due to expression of other pili (Table 1) or other putative adhesins present in the genome (results not shown). Differences in the attenuation of pilus mutants according to the route of challenge have been reported previously with *Yersinia pseudotuberculosis* (8). As bacterial adhesins often recognize specific receptors (20, 34, 37), attenuation differences for JAB16 due to route of challenge may be due to differential distribution and/or expression of the cognate host receptor.

Concluding remarks. The identification of eight loci encoding a total of seven putative type IV pilin subunits, and many accessory genes, may be related to the capacity of *B. pseudomallei* to exist and replicate in the environment and infect various animal hosts and tissues. It is possible that different pili are required to mediate interactions with specific host receptors or that the expression of *B. pseudomallei* pili is regulated in a complex manner. Work to further characterize the role of TFP loci is in progress.

An unmarked in-frame deletion of *pilA* in *B. pseudomallei* decreases adherence to cultured respiratory cell lines, decreases the time to death of *C. elegans*, and reduces the killing of BALB/c mice. Since the mutation in JAB16 is an in-frame deletion, it should not affect the expression of downstream genes, making it highly likely that the phenotypes observed for JAB16 are due to the absence of *pilA*. Our data suggest

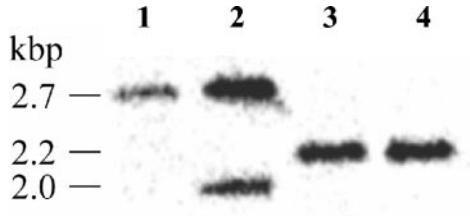

FIG. 2. Southern hybridization of ClaI-digested genomic DNA with the use of a *pilA*-specific DNA probe. Lanes: 1, the hybridizing probe identified a 2.7-kbp fragment from K96243 DNA containing the wild-type *pilA* allele; 2, the hybridizing probe identified two fragments from JAB16.1x DNA, one fragment containing the wild-type *pilA* allele (2.7 kbp) and the other containing the deleted *pilA* allele (2.0 kbp); 3 and 4, the hybridizing probe identified only the DNA fragment containing the deleted *pilA* allele (2.2 kbp) from DNA isolated from two individual putative deletion mutants (one designated JAB16). The 2.2-kbp fragment in the deletion mutant strains is larger than the 2.0-kbp fragment in JAB16.1x, as the excised plasmid contained a ClaI site.

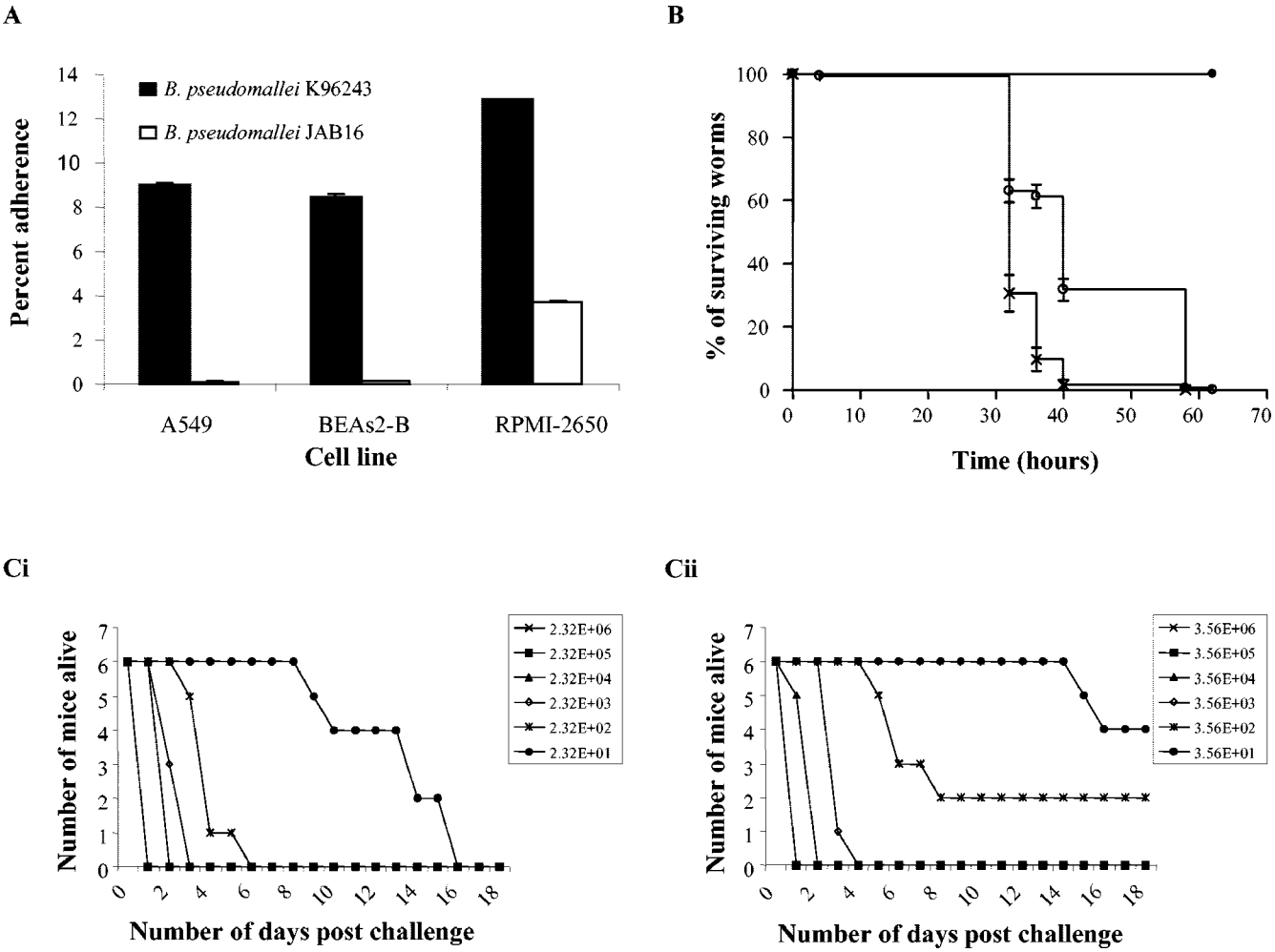

FIG. 3. Characterization of JAB16. (A) Adherence of *B. pseudomallei* K96243 and JAB16 to human epithelial cell lines. Percent adherence was determined by dividing the number of adherent bacteria by the inoculum and multiplying by 100. Percent adherence for the RPMI-2650 cell line was multiplied by 100. Data represent the means ± standard errors of the means for triplicate wells of single representative experiments. Each experiment was performed at least three times. The independent sample *t* test was used to analyze differences in cell-associated bacteria between K96243 and JAB16. (B) Killing of *C. elegans* by *B. pseudomallei* K96243 and JAB16. Between 10 and 20 *C. elegans* L4-stage worms were seeded onto nematode growth medium agar, which was inoculated with 10 μl of an overnight culture of *B. pseudomallei* K96243 (×) or *B. pseudomallei* JAB16 (○). *E. coli* OP50 (●) was used as a negative control. Each value is the mean ± standard error of the mean of six replicates. The survival data were plotted using the Kaplan-Meier method and analyzed using the Mantel-Haenszel log rank test in the statistical package Prism, version 3.02. (C) Survival curves of BALB/c mice challenged with different doses of *B. pseudomallei* K96243 (Ci) and JAB16 (Cii), by the intranasal route of infection. Infected BALB/c mice (groups of six) were monitored for 18 days after bacterial challenge. By the use of regression with life data *B. pseudomallei* JAB16 is attenuated with respect to *B. pseudomallei* K96243 ($P = 0.012$).

that PilA may be an important mediator of the pathogenic process in humans and should be considered as a target in future attempts to generate a protective vaccine against melioidosis.

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