Identification of the acid phosphatase (*acpA*) gene homologues in pathogenic and non-pathogenic *Burkholderia* spp. facilitates *TnphoA* mutagenesis

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***Burkholderia pseudomallei* and *Burkholderia mallei* are pathogens responsible for disease in both humans and animals. *Burkholderia thailandensis*, while phylogenetically similar, is considered avirulent in comparison. These three species exhibit phosphatase activity when grown on media containing chromogenic substrates such as 5-bromo-4-chloro-3-indolyl phosphate (XP). Tn5-OT182 mutagenesis has been utilized to isolate mutants of *B. pseudomallei* and *B. thailandensis* unable to hydrolyse XP. Sequence analysis of these mutants revealed an ORF of 1734 nucleotides demonstrating a high degree of homology to the *acpA* gene product of *Francisella tularensis*. PCR primers were designed based on the *B. pseudomallei acpA* gene sequence and were used to amplify an *acpA* homologue from *B. mallei*. The predicted amino acid sequence of *B. pseudomallei* AcpA differed from those of the predicted *B. thailandensis* AcpA and *B. mallei* AcpA by 15 and 3 amino acids, respectively. Allelic exchange was used to construct $\Delta acpA$ mutants in each of these *Burkholderia* spp. These mutants were shown to be devoid of phosphatase activity and have subsequently allowed for the implementation of *phoA* fusion transposon mutagenesis systems. Two such systems have been successfully utilized in *Burkholderia* spp. for the identification of several genes encoding exported proteins.**

Keywords: *Burkholderia* spp., acid phosphatase, *TnphoA*, exported proteins

INTRODUCTION

Burkholderia pseudomallei, *Burkholderia thailandensis* and *Burkholderia mallei* are three closely related Gram-negative bacteria. *B. pseudomallei* is the causative agent of melioidosis, a disease endemic to South-east Asia and Northern Australia (Smith *et al.*, 1987) while *B. mallei* is the causative agent of glanders, an equine zoonosis (Arun *et al.*, 1999). *B. thailandensis* is an avirulent species that is genetically very similar to *B. pseudomallei* and *B. mallei*; however, it lacks at least one pathogenicity island present in these species (Brett *et al.*, 1998; Reckseidler *et al.*, 2001). Melioidosis and glanders have

relatively high mortality rates and studies to elucidate the factors that contribute to their pathogenesis are necessary. *B. thailandensis* is a particularly useful laboratory tool for genetic manipulations under avirulent conditions which may contribute to the understanding of functions common to *B. pseudomallei* and *B. mallei*.

B. pseudomallei and *B. mallei* synthesize a variety of secreted enzymes (DeShazer *et al.*, 1999) and surface antigens; however, the roles of such factors in the pathogenesis of the diseases caused by these organisms remain poorly defined. To define the role(s) of particular exported proteins in pathogenesis, it is necessary to employ a system in which defined mutations can be made in genes encoding such products. The system we have chosen to investigate and implement in this study is the *TnphoA* fusion vector system. The *phoA* gene fusion approach relies on the fact that the periplasmic bacterial

Abbreviations: AP, acid phosphatase; XP, 5-bromo-4-chloro-3-indolyl phosphate.

The GenBank accession numbers for the sequences reported in this paper are AF252862, AF252863 and AF276770.

alkaline phosphatase (PhoA) must be located extra-cytoplasmically for enzymic activity to occur (Taylor *et al.*, 1989; Manoil & Beckwith, 1985). *TnphoA* utilizes a Tn5 transposon containing a truncated *phoA* gene which lacks a signal sequence; this transposon can generate *phoA* gene fusion randomly upon integration into the recipient bacterial chromosome (Taylor *et al.*, 1989; Manoil & Beckwith, 1985). If the targeted gene encodes an exported protein then the hybrid protein expressed will exhibit PhoA activity and the resulting colony will appear blue when grown on medium containing the chromogenic substrate 5-bromo-4-chloro-3-indoyl-phosphate (XP). Due to the fact that exported proteins are frequently involved in pathogenesis, this system provides a means by which the selection for the identification of virulence genes is enhanced. There are a number of instances in the literature in which *TnphoA* mutagenesis has been used successfully for the identification of virulence factors. Some examples include involvement of OmpA in the virulence in *Escherichia coli* K-1 (Weiser & Gotschlich, 1991), identification of OMPs in the pathogenesis of *Salmonella abortusovis* (Rubino *et al.*, 1993), characterization of virulence genes of enteroinvasive *E. coli* (Hsia *et al.*, 1993), recognition that *TnphoA* mutants in penicillin-binding proteins from *Erwinia amylovora* are avirulent (Milner *et al.*, 1993) and identification of antigens involved in colonization of *Vibrio cholerae* O139 (Bondre *et al.*, 1997).

B. pseudomallei exhibits phosphatase activity when grown on agar containing XP. To implement a *phoA* gene fusion system in *B. pseudomallei*, a strain that cannot hydrolyse XP must be utilized. It is known that some of this phosphatase activity is due to a surface-bound glycoprotein possessing acid phosphatase (AP) activity (Kanai & Kondo, 1994; Kondo *et al.*, 1996). However, the gene encoding an AP has remained unidentified prior to this study. In the present study we describe the sequence of the AP (*acpA*) gene homologues present in *B. pseudomallei*, *B. thailandensis* and *B. mallei*. The AP activity associated with the *acpA* gene product was assessed. The inactivation of the *acpA* gene homologues and subsequent complementation confirms that the *acpA* gene product is responsible for the AP activity present in these species. In addition, strains harbouring disrupted *acpA* gene homologues were constructed and have allowed for mutagenesis using *TnphoA* (Manoil & Beckwith, 1985) and mini-*OphoA* (Bolton & Woods, 2000) for the identification of genes involved in the production of exported proteins in these *Burkholderia* spp.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *B. pseudomallei* and *B. thailandensis* cultures were incubated at 37 °C on Luria-Bertani (LB) agar plates or in LB broth with agitation at 250 r.p.m. *B. mallei* cultures were grown at 37 °C on tryptic soy agar supplemented with 4% glycerol (TSG) or in TSG broth. Antibiotics were purchased

from Sigma and Invitrogen. For *E. coli*, antibiotics were used at the following concentrations: 100 µg ampicillin (Ap) ml⁻¹, 25 µg kanamycin (Km) ml⁻¹, 25 µg chloramphenicol (Cm) ml⁻¹, 100 µg streptomycin (Sm) ml⁻¹, 15 µg gentamicin (Gm) ml⁻¹, 15 µg tetracycline (Tc) ml⁻¹, 50 µg polymyxin B (Pm) ml⁻¹, 1.5 mg trimethoprim (Tp) ml⁻¹ and 25 µg zeocin (Ze) ml⁻¹. For *B. pseudomallei* and *B. thailandensis* the antibiotic concentrations used were 50 µg Km ml⁻¹, 50 µg Tc ml⁻¹, 100 µg Tp ml⁻¹ and 100 µg Ze ml⁻¹ unless otherwise stated. For *B. mallei* antibiotic concentrations used were 75 µg naladixic acid (Nx) ml⁻¹, 5 µg Km ml⁻¹, 5 µg Ze ml⁻¹, 15 µg Pm ml⁻¹ and 5 µg Gm ml⁻¹.

Plasmids were purified using the Concert rapid plasmid miniprep system (GibcoBRL), QIAprep spin plasmid miniprep kit (Qiagen) or QIAprep midipreps for plasmid DNA (Qiagen).

Tn5-OT182 mutagenesis and screening. *B. pseudomallei* 1026b and *B. thailandensis* E264 were mutagenized with Tn5-OT182 as previously described (DeShazer *et al.*, 1997). *B. pseudomallei* conjugations were incubated at 37 °C for 8 h while those of *B. thailandensis* were incubated at 37 °C for 2 h. Transconjugants were selected for on LB agar plates containing 100 µg Sm ml⁻¹ and 50 µg Tc ml⁻¹ with 40 µg XP ml⁻¹. White colonies were retained for further analyses.

DNA manipulation and transformations. Restriction endonucleases and T4 DNA ligase were purchased from GibcoBRL and New England Biolabs, respectively, and were used according to the manufacturers' instructions. DNA fragments excised from agarose gels and used in cloning procedures were purified using a QIAquick gel extraction kit (Qiagen). A Wizard genomic DNA purification kit (Promega) was used for isolation of genomic DNA from bacterial strains. The DNA immediately flanking Tn5-OT182 integrations was self-cloned as previously described (DeShazer *et al.*, 1997). In brief, approximately 5 µg chromosomal DNA from Tn5-OT182 mutants was digested with restriction enzyme, boiled for 5 min and precipitated with 0.1 vol. 3 M sodium acetate and 2 vols 100% ethanol. This mixture was placed at -70 °C for at least 30 min, centrifuged and washed with 70% ethanol. The resulting DNA was air-dried, resuspended in distilled water and ligation reactions were prepared. Transformations were performed with 2–10 µl ligation mixture using chemically competent *E. coli* cells.

Phosphatase activity assays. AP activity assays were performed in triplicate using a previously described method (Kondo *et al.*, 1991b, 1996). Supernatants, periplasmic proteins and whole cells were prepared from 1 ml of overnight cultures grown at 37 °C. Supernatants were harvested and filter-sterilized through a 0.22 µm filter (Millipore) for use in supernatant assays. Whole cells were pelleted, resuspended in 1 ml 0.01 M Tris/HCl pH 8.0 and used in whole-cell assays. Periplasmic proteins were extracted using a previously described chloroform extraction method (Ames *et al.*, 1984). In a typical assay, 20 µl of the test sample, 20 µl *p*-nitrophenyl phosphate (0.2%, w/v, solution) and 160 µl 0.1 M sodium acetate buffer pH 5.5 were mixed and incubated at 37 °C for 30 min in microtitre wells. Then 100 µl 0.5 M NaOH was added and the colour was allowed to develop for 5 min. Plates were read at 405 nm.

PCR amplification and cloning of PCR products. The *acpA* gene homologues were amplified from *B. pseudomallei* 1026b and *B. mallei* ATCC 23344 chromosomal DNA via PCR. The oligodeoxyribonucleotide primers used were AP-5' (GCTCTAGACGAGCGGACGGGAAATGGCG) contain-

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1</i>	Bethesda Research Laboratories
Top 10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 deoR recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Sm ^R) <i>endA1 nupG</i>	Invitrogen
SM10 λ pir	SM10 with a λ prophage carrying the gene	Miller & Mekalanos (1988)
<i>B. pseudomallei</i>		
1026b	Clinical isolate (human); Km ^R Gm ^R Pm ^R Sm ^R Tc ^S	DeShazer <i>et al.</i> (1997)
DD503	1026b derivative; allelic exchange strain; Δ (<i>amrR-oprA</i>); Km ^S Gm ^S Sm ^S <i>rpsL</i> Sm ^R	Moore <i>et al.</i> (1999)
APM402	1026b derivative; ORF::Tn5-OT182	This study
APM403	1026b derivative; <i>apcA</i> ::Tn5-OT182	This study
APM403C	APM403 (p29 <i>acpA</i>)	This study
MB401Z	DD503 derivative; <i>acpA</i> Δ (nt 1–1139):: <i>shble</i> -p15Aori; Ze ^R	This study
MB401	DD503 derivative; <i>acpA</i> Δ (nt 1–1139)	This study
<i>B. thailandensis</i>		
E264	Environmental isolate; Km ^R Gm ^R Pm ^R Sm ^R Tc ^S	Brett <i>et al.</i> (1998)
DW503	E264 derivative; allelic exchange strain; Δ (<i>amrR-oprA</i>); Km ^S Gm ^S Sm ^S ; <i>rpsL</i> Sm ^R	This study
APM501	E264 derivative; <i>udp-rfaH</i> ::Tn5-OT182	This study
APM502	E264 derivative; <i>apcA</i> ::Tn5-OT182	This study
APM501C	APM501 (p29 <i>acpA</i>)	This study
DW401Z	DW503 derivative; <i>acpA</i> Δ (nt 1–1139):: <i>shble</i> -p15Aori; Ze ^R	This study
DW401	DW503 derivative; <i>acpA</i> Δ (nt 1–1139)	This study
<i>B. mallei</i>		
ATCC 23344 (formerly GB8)	Clinical isolate (equine)	USAMRIID
G8N	ATCC 23344 derivative; Nx ^R	This study
G8P	ATCC 23344 derivative; <i>acpA</i> Δ (nt 1–1139):: <i>shble</i> -p15oriV; Ze ^R	This study
G8PN	G8P derivative; Ze ^R Nx ^R	This study
Plasmids		
pOT182	pSUP102(Gm)::Tn5-OT182; Cm ^R Gm ^R Ap ^R Tc ^R	Merriman & Lamont (1993)
pUC19	Cloning vector	New England Biolabs
pCR2.1TOPO	Cloning vector; pUC ori; Ap ^R Km ^R	Invitrogen
pUCP29T	Broad-host-range vector; IncP OriT; pRO1600 ori; Tp ^R	Schweizer <i>et al.</i> (1996)
p34EoriZeo	Vector containing self-cloning Ze ^R cassette; <i>shble</i> -p15oriV; this vector was derived from p34Eori and the Zeo cassette from pEM7/Zeo (Invitrogen)	P. J. Brett, D. DeShazer, M. N. Burtnick & D. E. Woods, unpublished
pKAS46	Allelic exchange vector; <i>rpsL</i> Sm ^S	Skorupski & Taylor (1996)
pAPM402E	0.7 kb <i>Eco</i> RI fragment from APM402 obtained by self-cloning; Ap ^R Tc ^R	This study
pAPM403E	8.0 kb <i>Eco</i> RI fragment from APM403 obtained by self-cloning; Ap ^R Tc ^R	This study
pAPM403H	5.5 kb <i>Hind</i> III fragment from APM403 obtained by self-cloning; Ap ^R Tc ^R	This study
pAPM501Ss	1.6 kb <i>Sst</i> I fragment from APM501 obtained by self-cloning; Ap ^R Tc ^R	This study
pAPM501H	6.5 kb <i>Hind</i> III fragment from APM501 obtained by self-cloning; Ap ^R Tc ^R	This study
pAPM502E	0.5 kb <i>Eco</i> RI fragment from APM502 obtained by self-cloning; Ap ^R Tc ^R	This study
pMB401	pUC19 containing 4.5 kb <i>Hpa</i> I/ <i>Sst</i> I fragment containing nt 1–1633 of the <i>acpA</i> gene	This study
pMB401X	pMB401 Δ (2.8 kb <i>Xho</i> I fragment)	This study
pMB401Z	pMB401 Δ (2.8 kb <i>Xho</i> I fragment); <i>shble</i> -p15AoriV; Ze ^R	This study
p46MB401X	pKAS46 containing 1.3 kb <i>Sst</i> I/ <i>Hind</i> III fragment from pMB401X	This study
p46MB401Z	pKAS46 containing 2.7 kb <i>Sst</i> I/ <i>Hind</i> III fragment from pMB401Z; Ze ^R	This study
p29 <i>acpA</i>	pUCP29T with 1.8 kb <i>Xba</i> I/ <i>Kpn</i> I fragment containing <i>B. pseudomallei acpA</i> gene	This study
pRT733	<i>oriR6K mobRP4 Tnp</i> <i>h</i> <i>o</i> A; Ap ^R Km ^R	Taylor <i>et al.</i> (1989)
pmini- <i>OphoA</i>	pMB1 <i>oriR</i> , Tn5 <i>tnp</i> , RP4 <i>oriT</i> , <i>phoA</i> ; Gm ^R	Bolton & Woods (2000)
pBR322	Cloning vector <i>oriColE1</i> ; Ap ^R Tc ^R	New England Biolabs

Fig. 1. (a) Plasmids containing *acpA* genes used in this study. Relative locations of Tn5-OT182 integrations in the *acpA* gene (black arrow) in AP-negative mutants, APM403 and APM501, are shown. Fragments obtained by self-cloning are shown, pAPM403E and pAPM403H. The fragment from pMB401 used for construction of strains for use in *TnphoA* mutagenesis is also shown; the 2.8 kb fragment deleted encompassing nucleotides 1–1139 of the *acpA* gene is shown as a thicker black line. (b) Scheme used for deletion of a portion of the *B. pseudomallei acpA* gene (black arrow on inside of plasmid) and subsequent construction of vectors for allelic exchange. Restriction sites are as follows: *EcoRI* (E), *HindIII* (H), *XhoI* (Xh), *SstI* (Ss), *HpaI* (Hp) and *BamHI* (B). The Hp and B sites were located on the ends of Tn5-OT182.

ing an *XbaI* linker and AP-3' (GGGGTACCTCTTGTCTACCGTACCGACC) containing a *KpnI* linker (linkers underlined). PCR amplification was performed in 100 µl reaction mixtures containing approximately 500 ng genomic DNA, 1 × PCR buffer (GibcoBRL), a 200 mM concentration of each deoxynucleoside triphosphate, a 0.5 mM concentration of each primer, 2 mM MgCl₂ (GibcoBRL), 1 × Q-solution (Qiagen) and 5 U *Taq* DNA polymerase (GibcoBRL) per µl. This mixture was placed in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus) thermal cycler and subjected to a 5 min denaturation step at 95 °C followed by 30 cycles at 95 °C for 45 s, 56 °C for 30 s and 72 °C for 90 s. The reaction mixture was next held at 72 °C for 10 min and then placed at 4 °C until analysed on a 1% agarose gel. The resulting PCR products were digested with *KpnI* and *XbaI* and cloned into pUC19 or cloned directly into pCR2.1TOPO (Invitrogen) using a TOPO TA Cloning Kit (Invitrogen). The cloned PCR products were sequenced on both strands.

Construction of allelic exchange mutants. Allelic exchange was performed in *B. pseudomallei* DD503 and *B. thailandensis* DW503 using the *rpsL*-based vector pKAS46 as previously described (Moore *et al.*, 1999; Skorupski & Taylor, 1996). Both DD503 and DW503 are Sm^s due to deletion of the *amrR*–*oprA* operon, but Sm^r due to a mutation in the *rpsL* gene (Moore *et al.*, 1999). Allelic exchange experiments in the present study employed the vectors p46MB401Z or p46MB401X, which were constructed using a deleted version of the *acpA* gene homologue from *B. pseudomallei*. The steps in construction of these vectors are described below and are also shown in Fig. 1(b). A 4.5 kb *SstI/HpaI* (Klenow-treated)

fragment containing nucleotides 1 to 1633 of the *acpA* gene was excised from pAPM403E and inserted into pUC19, creating pMB401. A 2.8 kb *XhoI* fragment was then excised from pMB401 and the vector was ligated back together with or without an oriZeo cassette, resulting in pMB401X or pMB401Z. Each of these fragments containing the *ΔacpA* gene homologue was separately inserted into pKAS46 to create p46MB401Z or p46MB401X. SM10 *λpir* strains containing these vectors were used in conjugation experiments with either DD503 or DW503. Transconjugants were selected for on LB agar containing 50 µg Pm ml⁻¹, 50 µg Km ml⁻¹ and 100 µg Ze ml⁻¹ when appropriate. Transconjugants were subsequently plated on 100 µg Sm ml⁻¹ alone or with 100 µg Ze ml⁻¹ to select for loss of the vector. These mutants were plated on LB agar plates containing 50 µg Km ml⁻¹ to confirm a double crossover event as indicated by lack of growth. Allelic exchange mutants were confirmed by Southern blot analysis.

A slightly different strategy was used for allelic exchange in *B. mallei*, because spontaneous Sm^r mutants of ATCC 23344 were also Sm dependent, i.e. these mutants could not grow in the absence of Sm. To overcome this problem, transconjugants from ATCC 23344 and SM10 *λpir*(p46MB401Z) mutagenesis were selected for on TSG agar containing 80 µg XP ml⁻¹, 15 µg Pm ml⁻¹ and 5 µg Ze ml⁻¹. One white transconjugant was Km^s, indicating loss of the vector (pKAS46) and thus, a double crossover event. This mutant was designated G8P. A spontaneously Nx^r derivative of G8P was selected for and obtained because Nx provided better selection than Pm. This Nx^r Ze^r XP-negative strain of *B. mallei* was designated G8PN and was used for further assessment.

Complementation of Tn5-OT182 mutants. A wild-type copy of the *acpA* gene obtained by PCR from *B. pseudomallei* 1026b DNA was cloned into the broad-host-range vector pUCP29T. This construct, designated p29*acpA*, was transformed into *E. coli* SM10 λ pir and conjugated to *B. pseudomallei* APM403 and *B. thailandensis* APM501 for 5 h, followed by selection on LB agar plates containing 100 μ g Sm ml⁻¹ and 100 μ g Tp ml⁻¹. The resulting strains were inoculated on similar LB agar plates with 40 μ g XP ml⁻¹ and blue colonies were retained.

TnphoA and mini-OphoA mutagenesis. In a typical TnphoA mutagenesis experiment, approximately 5 μ l of an overnight culture of SM10 λ pir(pRT733) containing TnphoA and 5 μ l of either *B. pseudomallei* MB401Z or *B. thailandensis* DW401Z were mixed together on an LB agar plate and incubated at 37 °C for 18 h. Eight to ten separate conjugations were carried out on a single plate concurrently along with donor and recipient alone as controls. Each individual conjugation was plated on a single agar plate. *B. pseudomallei* and *B. thailandensis* transconjugants were selected for on LB agar containing 300 μ g Km ml⁻¹, 100 μ g Sm ml⁻¹, 100 μ g Ze ml⁻¹ and 40 μ g XP ml⁻¹. For *B. mallei* G8PN a similar procedure was employed except that transconjugants were selected for on TSG agar plates containing 5 μ g Km ml⁻¹, 75 μ g Nx ml⁻¹, 5 μ g Ze ml⁻¹ and 80 μ g XP ml⁻¹. Plates were incubated at 37 °C for 48 h and blue colonies were retained for further analysis. The DNA immediately flanking the TnphoA integration was cloned as previously described (Taylor *et al.*, 1989) using the cloning vector pBR322 and BamHI- or SalI-digested genomic DNA. The resulting plasmids were sequenced using a previously described primer sequence (Taylor *et al.*, 1989).

Mini-OphoA was constructed using the Tn5-based plasposon pTnModOGm (Dennis & Zylstra, 1998) and the *phoA* gene from pRT733 (TnphoA) (Manoil & Beckwith, 1985). Mini-OphoA is small (3.4 kb) and contains an origin of replication that allows for self-cloning of the chromosomal DNA adjacent to transposon integrations (Bolton & Woods, 2000). *B. pseudomallei* MB401, *B. thailandensis* DW401 and *B. mallei* G8PN strains were recipient strains for mini-OphoA mutagenesis experiments. Conjugations were performed as described for TnphoA using 5 μ l of donor and recipient strains on LB or TSG agar plates at 37 °C for 18 h. Transconjugants of *B. pseudomallei* and *B. thailandensis* were selected for on LB agar plates containing 100 μ g Sm ml⁻¹, 15 μ g Gm ml⁻¹ and 40 μ g XP ml⁻¹. *B. mallei* transconjugants were selected for on TSG agar plates containing 5 μ g Gm ml⁻¹, 75 μ g Nx ml⁻¹, 5 μ g Ze ml⁻¹ and 80 μ g XP ml⁻¹. Self-cloning of the DNA immediately flanking mini-OphoA integrations was performed essentially as previously described for Tn5-OT182 (DeShazer *et al.*, 1997). Briefly, genomic DNA of mutants harbouring mini-OphoA was isolated then digested with NotI at 37 °C for 1 h. These reactions were then heat-inactivated followed by ethanol precipitation. Ligation reactions were set up for 1 h at room temperature or overnight at 16 °C then transformed into chemically competent *E. coli* DH5 α or Top 10 cells. The resulting plasmids were isolated and sequenced.

DNA sequencing and analysis. DNA sequencing was performed by University Core DNA Services (University of Calgary). The previously described oligodeoxynucleotide primers OT182-RT and OT182-LT (DeShazer *et al.*, 1997) were used for sequencing of plasmid DNA obtained by self-cloning of Tn5-OT182 mutants. The previously described primer sequence (5'-AATATCGCCCTGAGC-3') was used for sequencing plasmids from TnphoA clones obtained in this study (Taylor *et al.*, 1989). Two deoxyoligonucleotide

primers, Pho-LT (5'-CAGTAATATCGCCCTGAGCAGC-3') and Gm-RT (5'-GCCGCGCAATTTCGAGCTC-3'), were used for sequencing the mini-OphoA clones (Bolton & Woods, 2000). Custom-designed primers were synthesized by University Core DNA Services and used in a primer walking strategy to obtain the sequence of both strands of the *acpA* gene homologue.

The DNA sequences obtained in this study were analysed using DNASIS v2.5 (Hitachi) and DIALIGN 2.1 (Morgenstern, 1999) for the presence of ORFs and restriction endonuclease cleavage sites, for sequence alignment and for translation to amino acid sequences. BLASTX and BLASTP programs were used to perform database searches in order to establish homology to known gene sequences (Altschul *et al.*, 1997).

The *acpA* gene sequences from *B. pseudomallei*, *B. thailandensis* and *B. mallei* were submitted to GenBank under accession nos AF252862, AF252863 and AF276770, respectively.

RESULTS

Identification of *acpA* homologues from *B. pseudomallei*, *B. thailandensis* and *B. mallei*

To identify and characterize the gene or genes responsible for the phosphatase activity exhibited by *B. pseudomallei*, we chose to employ Tn5-OT182 mutagenesis in combination with a simple screen in which the chromogenic substrate XP was incorporated into LB agar plates. Approximately 7000 *B. pseudomallei* Tn5-OT182 mutants were plated onto media containing XP. Two mutants were identified that were unable to hydrolyse XP as indicated by their lack of blue colour. These two *B. pseudomallei* phosphatase-negative mutants were designated APM402 and APM403. The DNA flanking the Tn5-OT182 integrations in each of these mutants was isolated by self-cloning using *Eco*RI and *Hind*III. The resulting plasmids were isolated and single-stranded sequence reactions were carried out. The sequences obtained were analysed using the BLASTX local alignment search tool. The sequence from APM403 demonstrated a high degree of homology to the AP of *Francisella tularensis* var. *novicida*. In contrast, the sequence from APM402 showed no significant homology to any sequences currently in the GenBank database.

The self-cloned plasmids, pAPM403E and pAPM403H (Fig. 1a), were sequenced for approximately 2 kb on each side of the Tn5-OT182 integration on both strands using a primer walking strategy. An ORF of 1734 nucleotides was identified. The product of this ORF demonstrated 36% similarity to the *acpA* gene of *F. tularensis* and was therefore designated the *B. pseudomallei acpA* gene homologue. PCR primers were designed based on this sequence in order to identify the *acpA* gene homologues of *B. thailandensis* and *B. mallei*, both of which exhibit phosphatase activity. This approach was successful for identification of the *B. mallei acpA* homologue, but ineffective for identifying the *B. thailandensis acpA* homologue. The PCR product obtained from *B. mallei* ATCC 23344 chromosomal DNA using the AP-5' and AP-3' primers migrated to the

Fig. 2. AP activities of the *B. pseudomallei*, *B. thailandensis* and *B. mallei* strains used in this study. Supernatant, periplasmic and whole-cell fractions were prepared from overnight cultures grown at 37 °C. See Methods for details. The values are means and standard deviations of a single experiment performed in triplicate.

same position as the *B. pseudomallei* 1026b PCR product on a 1% agarose gel; both were approximately 1.8 kb in size. This result indicated that the *acpA* gene homologues present in both *B. pseudomallei* and *B. mallei* were probably very similar. These products were cloned and subjected to sequence analysis, which confirmed them to be *acpA* homologues. The sequence of the *B. mallei acpA* homologue was then completed on both strands.

Since we were unable to obtain a *B. thailandensis acpA* homologue by PCR, we chose to employ Tn5-OT182 mutagenesis to isolate this gene. Approximately 5000 Tn5-OT182 mutants of *B. thailandensis* E264 were plated onto LB agar plates containing XP and two white mutants were obtained. These mutants were designated APM501 and APM502. The chromosomal DNA immediately flanking the Tn5-OT182 integrations in these mutants was obtained by self-cloning using *Sst*I and *Hind*III. The sequences obtained from pAPM501S and pAPM501H demonstrated highest homology to the *B. pseudomallei acpA* gene homologue. Primer walking was employed to sequence the *B. thailandensis acpA* homologue on both strands. The sequence from the *B. thailandensis* APM502 was shown to have highest homology to the UDP-*rfaH* intergenic region of *E. coli*.

AP activity of *B. pseudomallei*, *B. thailandensis* and *B. mallei* strains

AP activity has previously been characterized for *B. pseudomallei* and it has been shown that optimal activity occurs at pH 5.5 and at 37 °C (Kanai & Kondo, 1994; Kondo *et al.*, 1991a, 1996). To confirm that the mutant strains isolated in this study lacked any AP activity, both the parent strains and the mutant strains were assayed as described in Methods (Fig. 2). All three

parent strains, *B. pseudomallei* 1026b, *B. thailandensis* E264 and *B. mallei* ATCC 23344, demonstrated similar levels of AP activity. The results of this assay indicated that the Tn5-OT182 mutants, APM403 and APM501, lacked any observable AP activity. In contrast, APM402 had considerable AP activity restricted to the periplasmic fraction and APM502 retained observable amounts of AP activity in both the periplasmic and whole-cell fractions.

Nucleotide sequence analysis of the *acpA* genes of *B. pseudomallei*, *B. thailandensis* and *B. mallei*

The nucleotide sequences of the *acpA* gene homologues from *B. pseudomallei* 1026b, *B. thailandensis* E264 and *B. mallei* ATCC 23344 demonstrated 23% identity and 36% similarity with the *acpA* gene product of *F. tularensis*. A 1734 bp ORF was identified, beginning with the ATG codon and ending with the TGA codon, that was consistent for *B. pseudomallei* and *B. mallei*, while a GTG start codon was identified for *B. thailandensis*. The G + C content of the *acpA* ORF was determined to be 69 mol%. A putative Shine–Dalgarno sequence was identified –6 to –10 bp upstream of the putative ATG/GTG start codons, suggesting that these are the correct start sites. The nucleotide sequences obtained for *B. thailandensis* and *B. mallei* were 94% and 99% identical, respectively, to the *B. pseudomallei acpA* sequence. The Tn5-OT182 integration associated with APM403 occurred at nucleotide 1633 of the *acpA* ORF while the Tn5-OT182 integration associated with APM501 occurred at nucleotide 365. The approximate positions are shown in Fig. 1(a). The putative protein encoded by *acpA* was predicted to be 578 amino acids in length with a calculated molecular mass of 62860 Da. Comparison of the predicted amino acid sequences of *B. thailandensis* AcpA and *B. mallei* AcpA to that of the *B. pseudomallei* AcpA predicted amino acid sequence revealed 15 and 3 differences, respectively. This reflects the close phylogenetic relationship between these species.

Characterization of AP-negative allelic exchange mutants

AP-negative mutants were constructed by allelic exchange as previously described with *B. pseudomallei* DD503 and *B. thailandensis* DW503 using the vectors p46MB401Z or p46MB401X (Fig. 1b). These vectors contained a portion of the *acpA* gene including nucleotides 1140–1633 along with 1.5 kb of upstream DNA that has not yet been sequenced. The *B. pseudomallei* Δ *acpA* mutants were designated MB401Z and MB401 and the *B. thailandensis* Δ *acpA* mutants were designated DW401Z and DW401. The strains MB401 and DW401 have a deletion in their *acpA* genes and lack the oriZeo marker present in the other AP-negative allelic exchange mutants, thus eliminating the need for the presence of Ze in selective media. As described in Methods, a slightly different strategy using the p46MB401Z vector was employed for allelic exchange in *B. mallei*. The

oriZeo cassette was used for positive selection in *B. mallei* allelic exchange and Ze was used in further experiments. The resulting Δ *acpA* strain of *B. mallei* was designated G8PN and was assessed for AP activity.

The isogenic allelic exchange mutant strains MB401Z/MB401, DW401Z/DW401 and G8PN were unable to hydrolyse the chromogenic substrate XP when present in LB or TSG agar. This was consistent with the observation that Tn5-OT182 disruptions in the *acpA* homologue caused APM403 and APM501 to display a white phenotype. Additionally, the allelic exchange mutants were essentially devoid of AP activity at pH 5.5 (Fig. 2) compared to wild-type strains. The inability of these strains to display blue colour when grown on agar containing XP made them good candidates as recipients for mutagenesis with Tn*phoA* and thus for the identification of exported products.

Complementation of AP-negative Tn5-OT182 strains

The 1.8 kb PCR product harbouring the *acpA* gene homologue *B. pseudomallei* was cloned into pUCP29T and was conjugated to *B. pseudomallei* APM403 and *B. thailandensis* APM501. The presence of p29*acpA* was able to restore the AP activity of these strains. The complemented strains, designated APM403C and APM501C, were blue when grown on LB agar plates containing XP and exhibited activity by AP activity assay (Fig. 2). These results indicate that the AP-negative phenotype observed in APM403 and APM501 is due to the Tn5-OT182 disruption in their *acpA* gene homologues and that this gene encodes a product that is responsible for the AP activity observed in these organisms. The strains constructed for Tn*phoA* mutagenesis were not complemented as the mutation encompasses 2.8 kb that has not been completely sequenced.

Tn*phoA* and mini-*OphoA* mutagenesis of *B. pseudomallei*, *B. thailandensis* and *B. mallei*

Two Tn5-based transposons containing truncated *phoA* genes were employed in this study. Initially, Tn*phoA* was delivered to MB401Z, DW401Z and G8PN on the vector pRT733 as previously described (Taylor *et al.*, 1989). This system worked efficiently for *B. pseudomallei* and *B. thailandensis*, resulting in approximately 1000–1200 Sm^R Km^R transconjugants per mutagenesis experiment, 1% of which were PhoA positive. However, in *B. mallei*, the Tn*phoA* transposition frequency was significantly lower: each mutagenesis resulted in only 50–200 Nx^R Km^R transconjugants with a frequency of PhoA-positive colonies of approximately 2%. Southern blot analysis using BamHI-digested chromosomal DNA from Tn*phoA* mutants confirmed that Tn*phoA* integrated only once per chromosome in four randomly selected *B. pseudomallei* and *B. thailandensis* PhoA-positive mutants.

Although this system is functional in these strains, the cloning procedures had a low efficiency, approximately

25%. This is suspected to be due in part to the size of the transposon and the fact that the cloning vector, pBR322, has a size limit on the DNA inserts that it can efficiently accept (approx. 7 kb). Upon digestion of the chromosomal DNA of PhoA-positive mutants with BamHI or SalI at least 5 kb of transposon remains along with the chromosomal DNA immediately flanking fragment. The cloning of DNA fragments containing Tn*phoA* and adjacent chromosomal DNA into pBR322 resulted in only relatively small (<2 kb) flanking DNA sequences being obtained. The resulting plasmids were sequenced and BLASTX searches were performed. Sequences showing significant homology over at least 300 bp of flanking DNA are shown in Table 2.

Due to the low numbers of transconjugants in *B. mallei* Tn*phoA* mutagenesis experiments, we chose to employ a second transposon, designated mini-*OphoA* (Bolton & Woods, 2000). The mini-*OphoA* system was found to be equivalent in transposition frequency to Tn*phoA* in *B. pseudomallei* and *B. thailandensis*, with the occurrence of PhoA-positive colonies being 0.5%. The transposition frequency was increased in *B. mallei*: approximately 1500 Ze^R Nx^R Gm^R transconjugants were obtained per mutagenesis, with the frequency of PhoA-positive colonies being 2%. In addition, due to the presence of an origin of replication, the cloning procedures for obtaining the DNA sequences flanking mini-*OphoA* integrations were simpler and more efficient than those for Tn*phoA*. Cloning efficiency was 90% when chemically competent *E. coli* Top 10 cells (Invitrogen) were used. The chromosomal DNA of six random Sm^R Gm^R transconjugants of *B. pseudomallei* and *B. thailandensis* and three random Ze^R Nx^R Gm^R transconjugants of *B. mallei* was isolated, digested with NotI and probed with α -³²P-labelled mini-*OphoA*. All nine mutants contained single copies of this transposon, suggesting that mini-*OphoA* integrates randomly into the chromosomes of *B. pseudomallei*, *B. thailandensis* and *B. mallei*.

The DNA from a number of PhoA-positive *B. pseudomallei*, *B. thailandensis* and *B. mallei* mini-*OphoA* mutants was self-cloned and subjected to single-stranded sequencing in order to characterize the DNA flanking the transposon integrations. Approximately 500–700 bp of sequence was obtained on each side of the mini-*OphoA* integrations. Subsequently, database searches were performed in order to establish homologies to known gene sequences. Some of the sequences obtained from the Pho-LT primer demonstrated significant homology over at least 300 bp and are shown in Table 2. A number of putative genes were identified which encoded proteins showing homology to secreted proteins, confirming the ability of this system to identify extracytoplasmic products expressed by the three *Burkholderia* spp. utilized in this study.

The DNA sequences adjacent to Tn*phoA* and mini-*OphoA* integrations in a number of PhoA-positive mutants did not show any significant homology to sequences currently in the GenBank database. These

Table 2. Table of homology of *TnphoA* and mini-*OphoA* flanking sequences

PhoA-positive mutant	Identity (%)	Similarity (%)	Homology	Entrez protein ID
<i>B. pseudomallei</i> <i>TnphoA</i> mutants				
PHOA8	40	43	Putative cell wall protein of <i>Streptomyces coelicolor</i>	AL137165
PHOA16	99	99	<i>gspG</i> (<i>B. pseudomallei</i>) type II secretion pathway gene	AAD05177.1
PHOA20	23	33	Hydroxyproline-rich glycoprotein of <i>Zea diploperennis</i>	228938
PHOA39	31	38	ExiT protein (exochelin ABC transporter) from <i>Mycobacterium smegmatis</i>	AAC32046.1
PHOA47	32	50	Outer-membrane protein C of <i>Pseudomonas aeruginosa</i>	BAA05664.1
<i>B. mallei</i> <i>TnphoA</i> mutant				
AJB34	75	95	Dipeptide transport system permease protein of <i>Escherichia coli</i>	AAC76568.1
<i>B. pseudomallei</i> mini-<i>OphoA</i> mutants				
PHOG4	33	43	Penicillin-binding protein of <i>Deinococcus radiodurans</i>	AAF10059.1
PHOG9	45	63	Periplasmic serine protease from <i>Aquifex aeolicus</i>	AAC07399.1
PHOG18	52	76	Putrescine-binding periplasmic protein precursor; permease protein from <i>Escherichia coli</i>	AAC73941.1
PHOG28	69	84	Phosphate-binding periplasmic protein precursor of <i>Erwinia carotovora</i>	AAB70458.1
PHOG29	37	50	Outer-membrane porin protein OpcP1 of <i>Burkholderia cepacia</i>	BAA09892.1
<i>B. thailandensis</i> mini-<i>OphoA</i> mutants				
PHOG103	36	44	Putative YME1 ATP-dependent zinc protease of <i>Arabidopsis thaliana</i>	AAC31223.1
<i>B. mallei</i> mini-<i>OphoA</i> mutant				
AJB101	43	56	Probable ABC transporter, permease protein of <i>Treponema pallidum</i>	F71375
AJB116	40	60	Branched-chain amino acid ABC transporter of <i>Deinococcus radiodurans</i>	H75444
AJB139	69	80	Phosphate-binding protein of <i>Enterobacter cloacae</i>	BAA22861.1
AJB150	36	50	Putative aromatic efflux pump outer-membrane protein of <i>Sphingomonas aromaticivorans</i>	AAD03862.1
AJB153	60	73	Periplasmic sorbitol-binding protein of <i>Rhodobacter sphaeroides</i>	AAC45766.1
AJB171	63	75	Outer-membrane protein C of <i>Pseudomonas aeruginosa</i>	BAA05664.1

sequences are of significant interest and may represent as yet undefined genes encoding exported products.

DISCUSSION

The AP activity of *B. pseudomallei* has been previously documented (Kanai & Kondo, 1994; Kondo *et al.*, 1991a, b, 1996); however, the gene responsible for this activity had not been identified prior to this study. The present work has demonstrated the presence of *acpA* gene homologues in *B. pseudomallei*, *B. thailandensis* and *B. mallei*. A simple screen was employed to identify mutants devoid of phosphatase activity. Several Tn5-OT182 mutants unable to hydrolyse XP were isolated and these subsequently facilitated the identification of *acpA* gene homologues in both *B. pseudomallei* and *B. thailandensis*. PCR primers designed from the nucleotide sequence of the *B. pseudomallei acpA* gene allowed the identification of an *acpA* homologue from *B. mallei*. The *acpA* genes from the species used in this study were

sequenced and the predicted amino acid compositions reflected the close phylogenetic relationship between these species. Complementation analyses have shown that functional *acpA* gene homologues are required for AP activity in these three *Burkholderia* spp. Furthermore, identification of *acpA* genes in these species has allowed the construction of strains with $\Delta acpA$ genes through allelic exchange.

The AP-negative strains constructed in this study have been used for mutagenesis experiments employing Tn5-based transposons containing truncated *phoA* genes. The *B. thailandensis* strain DW401/DW401Z will be particularly useful as it is a non-virulent strain that can be used as a laboratory tool for the identification of genes likely to be present in the highly virulent, closely related *B. pseudomallei* and *B. mallei* strains. The results of this study clearly indicate that Tn5-based transposons containing truncated *phoA* genes can be efficiently used in *B. pseudomallei* and *B. thailandensis* strains. It is not clear why *TnphoA* mutagenesis was not effective in *B.*

mallei; it may be due to an incompatibility with the vector carrying the transposon. However, this problem was overcome by employing a second transposon system, mini-*Op*hoA, that was shown to integrate efficiently in this species.

PhoA-positive transposon mutants have been isolated in this study and sequence analysis of DNA flanking transposon insertions has revealed homology to a number of known gene sequences. We have demonstrated that the *phoA* fusion approach can be efficiently used in *Burkholderia* spp. for the identification of genes encoding exported proteins. The *phoA* systems employed in this study have facilitated the identification of genes potentially contributing to the pathogenesis of melioidosis and glanders. We are currently constructing isogenic mutants in specific genes identified via *phoA* mutagenesis; this will allow for the assessment of the contribution of particular genes to the phenotypes displayed by these organisms. Such mutants will be used in virulence testing. This will help to establish the roles specific exported products play in pathogenesis.

Preliminary studies on the role of the *acpA* gene product in the pathogenesis of *B. pseudomallei* and *B. mallei* infections indicate that the disruption of the *acpA* gene does not significantly alter virulence (data not shown). The mutants harbouring disrupted *acpA* genes may be useful for future studies regarding the specific functioning of the *acpA* gene and its product. Identification of the *acpA* gene and the subsequent implementation of *phoA* mutagenesis systems described in the present study will contribute to the continuing studies on the pathogenesis of melioidosis and glanders.

ACKNOWLEDGEMENTS

This research is supported by the Canadian Bacterial Diseases Network Centers for Excellence. M. N. B. is the recipient of an Alberta Heritage Foundation for Medical Research (AHFMR) studentship. D. W. is the recipient of an AHFMR summer studentship.

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Received 27 June 2000; revised 12 September 2000; accepted 5 October 2000.