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

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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-indolyl-phosphate (XP). Due to the fact that
exported proteins are frequently involved in patho-
genesis, 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), charac-
terization 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
aladaxic 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 endo-
nuclease and T4 DNA ligase were purchased from GibcoBRL
and New England Biolabs, respectively, and were used
according to the manufacturers’ instructions. DNA fragments
cut 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 vol. 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 de-
scribed chloroform extraction method (Ames et al., 1984). In
a typical assay, 20 µl of the test sample, 20 µl p-nitrophenol
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
(GCTCTAGACGAGCGGACGGGAAGATGGCG) contain-
### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
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<tr>
<td><strong>Strains</strong> <strong>E. coli</strong></td>
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<td>DH5α</td>
<td>F− 80sdlacZ ΔM15 ΔlacZYA–argF(U169 endA1 recA1 hisdR17 deoR thi-1 supE44 gyrA96 relA1</td>
<td>Bethesda Research Laboratories</td>
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<td>Top10</td>
<td>F− mcrA Δ(mrr–brsRRM–mrcBC) 80sdlacZ ΔlacX74 deoR recA1 araD139 Δ(ara–leu)7697 galU galK rpsL (SmR) endA1 mupG</td>
<td>Invitrogen</td>
</tr>
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<td>SM10 3pir</td>
<td>SM10 with a λ prophage carrying the gene</td>
<td>Miller &amp; Mekalanos (1988)</td>
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<td><strong>B. pseudomallei</strong></td>
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<td></td>
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<td>1026b</td>
<td>Clinical isolate (human); KmR GmR PmR SmR TeR</td>
<td>DeShazer et al. (1997)</td>
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<td>DD503</td>
<td>1026b derivative; allelic exchange strain; Δ(rpsL) SmR</td>
<td>Moore et al. (1999)</td>
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<td>APM402</td>
<td>1026b derivative; ORF:::Tn5-OT182</td>
<td>This study</td>
</tr>
<tr>
<td>APM403</td>
<td>1026b derivative; apcA:::Tn5-OT182</td>
<td>This study</td>
</tr>
<tr>
<td>APM403C</td>
<td>APM403 (p29acpA)</td>
<td>This study</td>
</tr>
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<td>MB401Z</td>
<td>DD503 derivative; apcAΔ(nt 1–1139):::shble-p15Aori; ZeR</td>
<td>This study</td>
</tr>
<tr>
<td>MB401</td>
<td>DD503 derivative; apcA Δ(nt 1–1139)</td>
<td>This study</td>
</tr>
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<td><strong>B. thailandensis</strong></td>
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<td>E264</td>
<td>E264 derivative; KmR GmR PmR SmR TeR</td>
<td>Brett et al. (1998)</td>
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<td>DW503</td>
<td>E264 derivative; allelic exchange strain; Δ(amrR–oprA); KmR GmR SmR; rpsL SmR</td>
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<td>E264 derivative; udp–rfaHΔ::Tn5-OT182</td>
<td>This study</td>
</tr>
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<td>APM502</td>
<td>E264 derivative; ΔrpsL SmR</td>
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<td>APM501 (p29acpA)</td>
<td>This study</td>
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<td>DW503 derivative; acpA Δ(nt 1–1139)</td>
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<td><strong>B. mallei</strong></td>
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<td>ATCC 23444 (formerly GB8)</td>
<td>Clinical isolate (equine)</td>
<td>USAMRIID</td>
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<td>ATCC 23444 derivative; Nxr</td>
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<td>G8P</td>
<td>ATCC 23444 derivative; acpAΔ(nt 1–1139):::shble-p15oriV; ZeR</td>
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<tr>
<td>G8PN</td>
<td>G8P derivative; ZeR Nxr</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<td>pOT182</td>
<td>pSUP102(Gm):::Tn5-OT182; CmR GmR ApR TcR</td>
<td>Merriman &amp; Lamont (1993)</td>
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<td>pUC19</td>
<td>Cloning vector</td>
<td>New England Biolabs</td>
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<td>pCR2.1TOPO</td>
<td>Cloning vector; pUC ori; ApR KmR</td>
<td>Invitrogen</td>
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<td>pUCP29T</td>
<td>Broad-host-range vector; IncP OriT; pRO1600 ori; TpR</td>
<td>Schweizer et al. (1996)</td>
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<td>p34EoriZeo</td>
<td>Vector containing self-cloning ZeR cassette; shble-p15oriV; this vector was derived from p34Eori and the Zeo cassette from pEM7/Zeo (Invitrogen)</td>
<td>P. J. Brett, D. DeShazer, M. N. Moore et al. (1996)</td>
</tr>
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<td>pKAS46</td>
<td>Alleric exchange vector; rpsL SmR</td>
<td>Skorupski &amp; Taylor (1996)</td>
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<td>pAPM402E</td>
<td>0.7 kb EcoRI fragment from APM402 obtained by self-cloning; ApR TcR</td>
<td>This study</td>
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<tr>
<td>pAPM403E</td>
<td>8.0 kb EcoRI fragment from APM403 obtained by self-cloning; ApR TcR</td>
<td>This study</td>
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<tr>
<td>pAPM403H</td>
<td>5.5 kb HindIII fragment from APM403 obtained by self-cloning; ApR TcR</td>
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<td>1.6 kb SsrI fragment from APM501 obtained by self-cloning; ApR TcR</td>
<td>This study</td>
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<tr>
<td>pAPMS501H</td>
<td>6.5 kb HindIII fragment from APM501 obtained by self-cloning; ApR TcR</td>
<td>This study</td>
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<tr>
<td>pAPMS502E</td>
<td>0.5 kb EcoRI fragment from APM502 obtained by self-cloning; ApR TcR</td>
<td>This study</td>
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<tr>
<td>pMB401</td>
<td>pUC19 containing 4.5 kb Hpal/SsrI fragment containing nt 1–1633 of the apcA gene</td>
<td>This study</td>
</tr>
<tr>
<td>pMB401X</td>
<td>pMB401 Δ(2.8 kb XhoI fragment)</td>
<td>This study</td>
</tr>
<tr>
<td>pMB401Z</td>
<td>pMB401 Δ(2.8 kb XhoI fragment); shble-p15AoriV; ZeR</td>
<td>This study</td>
</tr>
<tr>
<td>p46MB401X</td>
<td>pKAS46 containing 1.3 kb SsrI/HindIII fragment from pMB401X</td>
<td>This study</td>
</tr>
<tr>
<td>p46MB401Z</td>
<td>pKAS46 containing 2.7 kb SsrI/HindIII fragment from pMB401Z; ZeR</td>
<td>This study</td>
</tr>
<tr>
<td>p29acpA</td>
<td>pUCP29T with 1.8 kb XbaI/KpnI fragment containing B. pseudomallei acpA gene</td>
<td>This study</td>
</tr>
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<td>pRT733</td>
<td>oriR6K mobRP4 TnphoA; ApR KmR</td>
<td>Taylor et al. (1989)</td>
</tr>
<tr>
<td>pmini-OpbOA</td>
<td>pMB1 oriR, Tn5 trp, RP4 oriT, phoA; GmR</td>
<td>Bolton &amp; Woods (2000)</td>
</tr>
<tr>
<td>pBR322</td>
<td>Cloning vector oriColE1; ApR TcR</td>
<td>New England Biolabs</td>
</tr>
</tbody>
</table>
Products were sequenced on both strands, using a TOPO TA Cloning Kit (Invitrogen). The cloned PCR product was either cloned into pUC19 or cloned directly into pCR2. 

Mixture was next held at 72°C for 5 min. 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, 5 mM concentration of each primer, 2 mM MgCl₂, and 5 U Taq DNA polymerase (GibcoBRL) were performed in 100 µl. This mixture was placed in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus) thermal cycle 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 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 and 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⁺ due to deletion of the amrR–oprA operon, but Sm⁻ due to a mutation in the rpsL gene (Moore et al., 1999). Allelic exchange experiments in the present study employed the vectors p46MB401X or p46MB401IX, 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 Δ*acpA* gene homologue was separately inserted into pKAS46 to create p46MB401X or p46MB401IX. SM10 *pir* 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⁻ 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 *pir* (p46MB401IX) 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⁻, indicating loss of the vector (pKAS46) and thus, a double crossover event. This mutant was designated G8P. A spontaneously Ns⁻ derivative of G8P was selected for and obtained because Ns provided better selection than Pm. This Ns⁻ Ze⁻ 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 p29acpA, was transformed into E. coli SM10 zip and conjugated to B. pseudomallei APM403 and B. thailandensis APM501 for 5 h, followed by selection on LB agar plates containing 100 µg Sm ml⁻¹ and 100 µg Tp ml⁻¹. The resulting strains were inoculated on similar LB agar plates with 40 µg XP ml⁻¹ and blue colonies were retained.

TpnoA and mini-Opoha mutagenesis. In a typical TpnoA mutagenesis experiment, approximately 5 µl of an overnight culture of SM10 zip(pRT733) containing TpnoA and 5 µl 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 µg Km ml⁻¹, 100 µg Sm ml⁻¹, 100 µg Ze ml⁻¹ and 40 µg XP ml⁻¹. For B. mallei G8PN a similar procedure was employed except that transconjugants were selected for on TSG agar plates containing 5 µg Km ml⁻¹, 75 µg Nx ml⁻¹, 5 µg Ze ml⁻¹ and 80 µg 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 TpnoA integration was cloned as previously described (Taylor et al., 1989) using the cloning vector pBR322 and BamHI or Sall-digested genomic DNA. The resulting plasmids were sequenced using a previously described primer sequence (Taylor et al., 1989).

Mini-Opoha was constructed using the Tn5-based plasmoson pTnoModOGm (Dennis & Zylstra, 1998) and the phoA gene from pRT733 (TpnoA) (Manoil & Beckwith, 1985). Mini-Opoha 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-Opoha mutagenesis experiments. Conjugations were performed as described for TpnoA using 5 µl 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 µg Sm ml⁻¹, 15 µg Gm ml⁻¹ and 40 µg XP ml⁻¹. B. mallei transconjugants were selected for on TSG agar plates containing 5 µg Gm ml⁻¹, 75 µg Nx ml⁻¹, 5 µg Ze ml⁻¹ and 80 µg XP ml⁻¹. Self-cloning of the DNA immediately flanking mini-Opoha integrations was performed essentially as previously described for Tn5-OT182 (DeShazer et al., 1997). Briefly, genomic DNA of mutants harbouring mini-Opoha 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 DH5α 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 oligodeoxyribonucleotide primers OT82-RT and OT82-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′-AATATCCGCTTGAGCGC-3′) was used for sequencing plasmids from TpnoA clones obtained in this study (Taylor et al., 1989). Two deoxyoligonucleotide primers, Pho-LT (5′-CAGTAATATCGGCCCTGAGCGC-3′) and Gm-R (5′-GCCGCGGCAATTCGAGCTC-3′), were used for sequencing the mini-Opoha 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 pBR322 and BamHI or Sall-digested genomic DNA. 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, pAM403E and pAM403H (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
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 mutants of B. thailandensis E264. 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 SstI and HindIII. The sequences obtained from pAPM501Ss 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-rhaH 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 ∆acpA mutants were designated MB401Z and MB401 and the B. thailandensis ∆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

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.
oriZeo cassette was used for positive selection in *B. mallei* allelic exchange and Ze was used in further experiments. The resulting ΔacpA 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 TnphoA and thus for the identification of exported products.

**Complementation of AP-negative Tn5-OT182 strains**

The 18 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 p29acpA 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 TnphoA mutagenesis were not complemented as the mutation encompasses 2–8 kb that has not been completely sequenced.

**TnphoA 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, TnphoA 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 SmR Kmr transconjugants per mutagenesis experiment, 1% of which were PhoA positive. However, in *B. mallei*, the TnphoA transposition frequency was significantly lower: each mutagenesis resulted in only 50–200 Nxr Kmr transconjugants with a frequency of PhoA-positive colonies of approximately 2%. Southern blot analysis using BamHI-digested chromosomal DNA from TnphoA mutants confirmed that TnphoA 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 TnphoA 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* TnphoA 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 TnphoA 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 ZeR NxR GmR 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 TnphoA. Cloning efficiency was 90% when chemically competent *E. coli* Top 10 cells (Invitrogen) were used. The chromosomal DNA of six random SmR GmR transconjugants of *B. pseudomallei* and *B. thailandensis* and three random ZeR NxR GmR transconjugants of *B. mallei* was isolated, digested with *N*otI and probed with α²P-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 TnphoA 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
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 Δ*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. pseudomallei*.

### Table 2. Table of homology of TnphoA and mini-OpHoA flanking sequences

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

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REFERENCES


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