Attenuated virulence and protective efficacy of a \textit{Burkholderia pseudomallei} \textit{bsa} type III secretion mutant in murine models of melioidosis

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Melioidosis is a severe infectious disease of animals and humans caused by the Gram-negative intracellular pathogen \textit{Burkholderia pseudomallei}. An Inv/Mxi-Spa-like type III protein secretion apparatus, encoded by the \textit{B. pseudomallei} \textit{bsa} locus, facilitates bacterial invasion of epithelial cells, escape from endocytic vesicles and intracellular survival. This study investigated the role of the Bsa type III secretion system in the pathogenesis of melioidosis in murine models. \textit{B. pseudomallei} \textit{bipD} mutants, lacking a component of the translocation apparatus, were found to be significantly attenuated following intraperitoneal or intranasal challenge of BALB/c mice. Furthermore, a \textit{bipD} mutant was attenuated in C57BL/6 IL-12 p40−/− mice, which are highly susceptible to \textit{B. pseudomallei} infection. Mutation of \textit{bipD} impaired bacterial replication in the liver and spleen of BALB/c mice in the early stages of infection. \textit{B. pseudomallei} mutants lacking either the type III secreted guanine nucleotide exchange factor BopE or the putative effectors BopA or BopB exhibited varying degrees of attenuation, with mutations in \textit{bopA} and \textit{bopB} causing a significant delay in median time to death. This indicates that \textit{bsa}-encoded type III secreted proteins may act in concert to determine the outcome of \textit{B. pseudomallei} infection in mice. Mice inoculated with the \textit{B. pseudomallei} \textit{bipD} mutant were partially protected against subsequent challenge with wild-type \textit{B. pseudomallei}. However, immunization of mice with purified BipD protein was not protective.

INTRODUCTION

\textit{Burkholderia pseudomallei} is the aetiologial agent of melioidosis, a severe invasive infection of humans and animals that is endemic in tropical and subtropical areas. Clinical signs of melioidosis can vary from inapparent or subacute infections to localized or chronic suppurative infections, which may progress to acute septicemia and pneumonia (reviewed by White, 2003). Latency and relapse are common even in melioidosis patients treated with appropriate antibiotics (Chaowagul \textit{et al.}, 1993). This may result in part from the ability of \textit{B. pseudomallei} to invade non-phagocytic host cells, and to survive and replicate within phagocytes, where antibiotics may be less effective (Jones \textit{et al.}, 1996; Kespichayawattana \textit{et al.}, 2000; Pruksachartvuthi \textit{et al.}, 1990). The severe course of infection, aerosol infectivity and worldwide availability of \textit{B. pseudomallei} has raised concerns that it may be used as a bioterror agent. No vaccine against melioidosis exists, and the molecular mechanisms underlying \textit{B. pseudomallei}–host cell interactions and virulence are incompletely understood (reviewed by Stevens & Galyov, 2004).

Recently, a type III protein secretion apparatus was identified in \textit{B. pseudomallei} (Bsa) that is similar to the \textit{Salmonella} Inv/Spa/Prg and \textit{Shigella} Ipa/Mxi/Spa systems (Attree & Attree, 2001; Rainbow \textit{et al.}, 2002; Stevens \textit{et al.}, 2002). Type III secretion systems (TTSSs) are key virulence determinants of \textit{Salmonella}, \textit{Shigella} and other Gram-negative facultative intracellular pathogens, and they serve to inject bacterial proteins into target cells (reviewed by Cornelis & van Gijsegem, 2000; Hueck, 1998; Galán, 2001;
B. pseudomallei et al. (2003) states that the B. pseudomallei bsa locus encodes homologues of Salmonella Sip translocator proteins (BipB, BipC and BipD) (Stevens et al., 2002). Salmonella SipB, SipC and SipD proteins are required for injection of effector proteins and invasion of epithelial cells in vitro (Collazo & Galán, 1997). In addition, SipD plays an important role in invasion of intestinal epithelium by Salmonella enterica serovar Dublin and the induction of enteritis (Bispham et al., 2001). Consistent with a role in the injection of effectors, mutation of the B. pseudomallei bipD gene impairs invasion of epithelial cells in vitro (Stevens et al., 2003). In addition, BipD is required for replication of B. pseudomallei in murine macrophage-like cells and for bacterial escape from endocytic vesicles and subsequent actin tail formation (Stevens et al., 2002). A protein encoded within the bsa locus (BopE) is secreted via the Bsa apparatus and influences invasion of HeLa cells, most likely by acting as a guanine nucleotide exchange factor for RhoGTPases that regulate the actin network (Stevens et al., 2003). Other putative bsa-encoded effector proteins have been identified, including BopA and BopB (Stevens et al., 2002). BopA is a homologue of the Shigella type III secreted protein IscB, which mediates cell-to-cell spread of Shigella by lysing the double membrane surrounding actin-based protrusions that project the bacteria into adjacent cells (Allaoui et al., 1992). BopB is predicted to be encoded at one end of the bsa locus by the seventh predicted gene downstream of bopA, and it contains an amino acid motif (CX2R) that is conserved in the catalytic domains of numerous phosphatases. A type III secreted protein of Salmonella containing a similar motif (SopB) influences inositol phosphate signalling pathways in eukaryotic cells, bacterial invasion and Salmonella-induced enteritis (Norris et al., 1998; Zhou et al., 2001).

The bsa locus is conserved in the glanders pathogen Burkholderia mallei, and two putative structural components of the type III secretion apparatus (BsaQ and BsaZ) were recently reported to be required for full virulence in rodent models of infection (Ulrich & DeShazer, 2004). The authors of that study did not examine the contribution of individual bsa-encoded translocator or effector proteins in pathogenesis, or the potential use of purified type III secreted proteins as subunit vaccines. TTSS components of other Gram-negative bacterial pathogens are protective antigens (Leary et al., 1995; Sawal et al., 1999). It is known that sera from convalescent melioidosis patients recognize the B. pseudomallei BipB, BipC and BipD proteins (Stevens et al., 2002); however, the protection offered by such responses has not been studied.

In this study, we investigated the role of BipD and known, or putative, bsa-encoded type III secreted effectors in B. pseudomallei virulence in murine models of melioidosis. In addition we examined the protective efficacy of the immune responses elicited by a B. pseudomallei bipD mutant strain and purified BipD protein.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** B. pseudomallei strains 576 and 10276 were isolated from fatal cases of human melioidosis in Thailand and Bangladesh, respectively, and they were obtained from Dr Ty Pitt, Health Protection Agency, Colindale, London, UK. K96243 is the genome-sequenced strain of B. pseudomallei originally isolated from a melioidosis patient in Thailand, and it was supplied by Dr S. Songsivilai of Mahidol University. Defined mutants of B. pseudomallei strain 10276 with insertions in bsaZ, bipD and bopE have been described previously (Stevens et al., 2002). Escherichia coli S17-1/pir (Simon et al., 1983) was used as a conjugative donor of the pir-dependent suicide replicon pDM4 (oriR6K, mobR4, sacBR, cat) and its derivatives (Milton et al., 1996). E. coli BL21(DE3) was used for expression of a BipD fusion protein from a pGEX-4T-1-based vector. Bacterial strains were grown using Luria–Bertani (LB) broth, LB agar or tryptone soy agar containing, as appropriate, 100 μg ampicillin ml⁻¹, 50 μg kanamycin ml⁻¹ or 50 μg chloramphenicol ml⁻¹. Bacterial strains were grown to stationary phase at 37 °C for 18–24 h, collected by centrifugation, then resuspended in phosphate-buffered saline (PBS) containing 20 % (v/v) glycerol, and frozen in 0.4 ml aliquots at a concentration of 5 x 10⁸ c.f.u. ml⁻¹ at -80 °C.

**Animals.** Female 7- to 10-week-old BALB/c and C57BL/6 IL-12 p40⁻/⁻ mice were housed under specific pathogen-free conditions on a 12 h light/12 h dark cycle with free access to food and water. All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986, and were approved by the local Ethical Review Committee.

**Mutagenesis of the B. pseudomallei bipD, bopA, bopB and bopE genes.** The genes encoding BipD and BopE and the putative type III secreted proteins BopA and BopB were disrupted by homologous recombination using pir-dependent suicide replicons. The pDM4-based constructs for disruption of bipD and bopE have been described (Stevens et al., 2002). They were introduced into B. pseudomallei strain 576 by conjugation from E. coli S17-1/pir. Insertion mutants (576 bipD::pDM4 and 576 bopE::pDM4) were selected by plating on medium containing kanamycin and chloramphenicol, and verified by PCR using pDM4- and gene-specific primers as described previously (Stevens et al., 2002).

To mutate bopA, an internal fragment of the gene was amplified by PCR using the oligonucleotides 5′-CGAAACACCTCGGAGGCGG- GCGGTTTCC-3′ and 5′-CGATGCAGATCGACGGCCGGG- GTTCGCC-3′ with Advantage GC2 DNA polymerase (Clontech) under optimal conditions for the amplification of GC-rich templates. The product was cloned into Xhol- and BglII-digested pDM4 via sites incorporated in the primers, and the construct was introduced into B. pseudomallei strain 576 from E. coli S17-1/pir as described above. A similar approach was used to mutate bopB. An internal fragment of the gene was amplified using the oligonucleotides 5′-GGGCCTCGGAGCGCGGCGG- GAGTTTCCGCC-3′ and 5′-CAGATCTCGTACATCAGT- GCCAGTGATCG-3′, the product was cloned into pDM4 opened...
with Xhol and BglII, and the construct was introduced into strain 576. Insertion mutants (576 hopA::pDM4 and 576 hopB::pDM4) were selected and verified as described above.

**Infection of animals.** For each infection, aliquots of *B. pseudomallei* wild-type or mutant strains were thawed from frozen stocks. Bacterial cells were diluted in PBS to the required concentration and administered either via the intraperitoneal route (0·2 ml) or via the intranasal route (0·05 ml), and mice were then monitored twice daily for symptoms of infection. Viable count determinations were performed to confirm the inoculation dose. Median lethal doses (MLDs) were determined by the method of Reed & Muench (1938). To enumerate bacteria in the liver and spleen, the organs were aseptically removed and homogenized in sterile PBS by passing them through a 100 μm mesh cell-strainer. Serial tenfold dilutions of tissue homogenates were plated onto tryptone soy agar, and colonies were enumerated after 24 h. Stability of pDM4 insertions in *bsa*-encoded genes was verified by plating of the recovered bacteria on LB medium, either with or without 50 μg chloramphenicol ml⁻¹.

**Immunization of BALB/c mice with purified BipD.** The pGEX-4T-1-based construct for expression of the translocator protein BipD as a glutathione-S-transferase (GST) fusion protein has been described previously (Stevens et al., 2002). Following expression in *E. coli* BL21(DE3) under isopropyl β-D-thiogalactoside induction, BipD-GST was purified using glutathione Sepharose 4B resin according to the Amersham Pharmacia GST gene fusion protocol, and BipD was cleaved from GST by digestion with thrombin. Purified BipD protein, or GST or PBS as controls, were separately administered on days 1, 14 and 28 to ten female BALB/c mice aged 7–9 weeks. For each immunization, 10 μg protein was given in 100 μl of 50 % (v/v) Ribi adjuvant (Corixa) in PBS by the intraperitoneal route. On day 35, the mice received 3·3 × 10⁶ c.f.u. *B. pseudomallei* strain K96243 by the intraperitoneal route, and they were monitored twice daily for the next 35 days. Expression of BipD by strain K96243 was confirmed by immunoblotting using BipD-specific rabbit polyclonal antiserum (data not shown).

**Detection of BipD-specific antibodies.** A 96-well ELISA plate was coated overnight at 4 °C with either 100 μl of 1 μg ml⁻¹ recombinant BipD in PBS or PBS control. Coated plates were washed three times with PBS plus 0·05 % Tween-20 (PBS-T). Plates were blocked with 5 % BSA in PBS, then washed with PBS-T as described above. Serum from animals immunized with BipD and PBS-treated controls (1/100 dilution in PBS) was added to an ELISA plate, serially diluted twofold in PBS, and then incubated at 37 °C for 60 min. After washing with PBS-T, HRP-conjugated secondary antibody, specific for mouse isotypes IgG1, IgG2a, IgG2b and IgG3 (Sigma), was added at a 1/2000 dilution to appropriate wells for 45 min. After washing, HRP activity was measured in a plate reader (414 nm absorbance) after 20 min incubation with 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid diammonium salt (ABTS) plus 0·02 % H₂O₂. The anti-BipD antibody titres for each IgG isotype were calculated as the maximum dilution of serum to give an A₄₁₄ reading 0·1 U greater than background, and they are presented as the reciprocal of the dilution.

**Statistical analysis.** Survival curves were compared using Log Rank tests, and *P* values <0·05 were taken to be significant.

**RESULTS**

**The *B. pseudomallei* BipD protein is required for full virulence in murine models of melioidosis.**

To assess the importance of BipD in pathogenesis, a defined bipD mutant was constructed using *B. pseudomallei* strain 576, which has been extensively characterized in the BALB/c mouse model (Atkins et al., 2002a, b). Consistent with earlier findings (Stevens et al., 2002, 2003), mutation of the bipD gene in *B. pseudomallei* strain 576 impaired invasion of HeLa cells and intracellular survival in J774.2 murine macrophage-like cells, and prevented actin tail formation (data not shown). No effect of the bipD mutation on the growth rate of strain 576 in LB medium was detected (data not shown). In initial experiments to assess the virulence of the mutant strain, six groups of five 7- to 9-week-old female BALB/c mice were inoculated via the intraperitoneal route with increasing doses (10⁻¹–10⁶ c.f.u.) of either the 576 wild-type or the 576 bipD::pDM4 mutant strain. At 35 days post-infection, the MLDs were calculated to be 80 c.f.u. for the *B. pseudomallei* 576 wild-type strain and 1·73 × 10⁴ c.f.u. for the 576 bipD::pDM4 mutant, indicating that the function of the Bsa TTSS is required for full virulence of *B. pseudomallei*.

Mutation of bipD was also found to be strongly attenuating following inoculation of mice via the intranasal route and when using a different *B. pseudomallei* strain. Groups of ten 8-week-old BALB/c mice were inoculated intranasally either with 10⁵ c.f.u. of strain 576 or 576 bipD::pDM4, or with 10⁴ c.f.u. of the previously described 10276 bipD::pDM4 mutant or the 10276 parent strain. All mice inoculated intranasally with 576 died within 6 days, whilst 7/10 mice given the 576 bipD::pDM4 mutant survived to day 47 (Fig. 1a). Bacteria were recovered from the spleens of 576 bipD::pDM4-infected mice that died on days 15 and 40 post-inoculation, and they were found to be exclusively chloramphenicol resistant, indicating that the insertion is stable in vivo. All mice given the 10276 bipD::pDM4 mutant survived, whilst mice given the 10276 strain died within 4 days (Fig. 1b). We also inoculated six mice intranasally with 10⁵ c.f.u. strain 10276 bsaZ::pDM4, which completely lacks the function of the TTSS (Stevens et al., 2003). All mice given this strain survived to 47 days post-inoculation (Fig. 1b). These observations further support our conclusion that a functional Bsa TTSS is required for full virulence of *B. pseudomallei* in mice. Mutation of bipD and bsaZ by pDM4 insertion is not expected to be polar, since the genes are predicted to be the last in their respective operons (Stevens et al., 2002).

**Attenuation of the *B. pseudomallei* bipD mutant in BALB/c mice correlates with reduced bacterial replication in the liver and spleen in the early phase of infection.**

Groups of twelve 7- to 9-week-old BALB/c mice were inoculated via the intraperitoneal route with either 10⁴ c.f.u. *B. pseudomallei* 576 wild-type or 10⁴ c.f.u. 576 bipD::pDM4. Three mice from each group were killed on days 1, 3, 5 and 7 post-inoculation, the liver and spleen were collected aseptically, and the number of viable bacteria per organ was enumerated by plating of serial dilutions of organ homogenates. No mice given the wild-type strain survived beyond 5 days post-inoculation. Identical numbers...
of the bipD mutant were recovered on medium with or without chloramphenicol, confirming the stability of the insertion. The spleens and livers of mice infected with the wild-type 576 strain were enlarged and contained multiple abscesses. Splenomegaly and abscess formation were less obvious with the 576 bipD::pDM4 (data not shown). In mice given the wild-type strain, rapid replication of the bacteria in the liver and spleen was detected; however, the load of the 576 bipD::pDM4 mutant in the spleen and liver was significantly lower at almost all time points (Fig. 2).

**B. pseudomallei** mutants lacking known or putative type III secreted effector proteins are attenuated in mice to differing extents

The finding that a functional Bsa TTSS is required for full virulence of *B. pseudomallei* in mice implies that the injection of bacterial effector proteins into host cells influences the outcome of infection. We therefore assessed the contribution of known or putative *bsa*-encoded type III secreted proteins to *B. pseudomallei* virulence in mice. Insertion mutants of strain 576 lacking *bopA*, *bopB* and *bopE* were constructed using *λpir*-dependent suicide plasmids. Mutation of *bopA*, *bopB* and *bopE* did not affect either the ability of *B. pseudomallei* to form actin tails following infection of J774.2 cells or the *in vitro* growth rate of the bacteria (data not shown). Groups of six 7- to 9-week-old BALB/c mice were inoculated via the intraperitoneal route with 5 × 10^4 c.f.u. 576 wild-type or one of the following mutant strains: 576 bipD::pDM4, 576 bopA::pDM4, 576 bopB::pDM4 or 576 bopE::pDM4. The median times to death for the wild-type and mutant strains were as follows: wild-type (20 days) < bopE (21 days) < bopB (30 days) < bopA (32 days) < bipD (50 days) (Fig. 3). Mutation of bipD significantly delayed time to death compared to the wild-type (*P* = 0.0005). However, all six mice given the 576 bipD::pDM4 mutant died by day 63, indicating that mutation of bipD slows, but does not

**Fig. 1.** Survival of BALB/c mice inoculated intranasally with (a) 1 × 10^2 c.f.u. *B. pseudomallei* 576 wild-type (□, 6 mice) or an isogenic 576 bipD::pDM4 mutant (○, 10 mice) or (b) 1 × 10^4 c.f.u. *B. pseudomallei* 10276 wild-type (■, 6 mice), an isogenic 10276 bipD::pDM4 mutant (◆, 10 mice), or an isogenic 10276 bsaZ::pDM4 mutant (▲, 6 mice). Mice were observed daily and percentage survival was plotted against time.

**Fig. 2.** Course of bacterial replication in the spleen (a) and liver (b) of BALB/c mice after inoculation with 1 × 10^4 c.f.u. *B. pseudomallei* strains 576 (■) or 576 bipD::pDM4 (○) by the intraperitoneal route. Three mice from each group were killed on days 1, 3, 5 and 7 post-inoculation. Values are given as the mean ± SEM.

**Fig. 3.** Survival of BALB/c mice inoculated via the intraperitoneal route with 5 × 10^4 c.f.u. 576 wild-type, isogenic 576 bipD::pDM4, 576 bopA::pDM4, 576 bopB::pDM4, 576 bopE::pDM4 mutant strains or PBS (6 mice per group).
abolish, the development of fatal melioidosis. Mutation of bopE did not cause a significant reduction in median time to death compared to the wild-type (P=0.59). Furthermore, we did not detect significant attenuation of either 576 bopE::pDM4 or 10276 bopE::pDM4 mutant following intranasal inoculation of groups of six BALB/c mice (data not shown). Mutation of bopA and bopB caused a significant delay in median time to death when compared to the bopE mutant (P=0.0155 for bopA, and 0.005 for bopB); however, the differences were not statistically significant when compared to the wild-type strain.

In this experiment, BALB/c mice inoculated via the intraperitoneal route with $5 \times 10^4$ c.f.u. 576 wild-type exhibited a longer median time to death than that observed in the experiments to determine MLD and the kinetics of replication in liver and spleen. Whilst these studies used the same dose, inoculation route and host, the experiments were performed at two independent laboratories and the discrepancy is likely to reflect differences in the source of animals and the humane end-point criteria applied. Regardless of such considerations, experiments with internal controls performed at either laboratory show a statistically significant reduction in B. pseudomallei virulence caused by mutation of bipD and bsaZ.

To assess the residual virulence of the bipD mutant in greater detail we infected C57BL/6 IL-12 p40$^{-/-}$ mice with the 576 wild-type and the bipD::pDM4 mutant strain. Mice in which interleukin (IL)-12 has been depleted by the administration of anti-IL-12 monoclonal antibody are acutely susceptible to B. pseudomallei infection (Santanirand et al., 1999). We inoculated groups of five C57BL/6 IL-12 p40$^{-/-}$ mice with $10^3$ c.f.u. strain 576, $10^4$ c.f.u. 576 bipD::pDM4 or PBS via the intraperitoneal route. The median time to death of C57BL/6 IL-12 p40$^{-/-}$ mice inoculated with the 576 bipD::pDM4 mutant was 13 days, compared to 6 days with the wild-type strain (P=0.0173) (Fig. 4).

**Infection of BALB/c mice with the B. pseudomallei bipD mutant partially protects against challenge with wild-type bacteria**

Survivors of the initial intraperitoneal challenges with the B. pseudomallei 576 bipD::pDM4 mutant were inoculated 5 weeks after the first inoculation with a matched dose of the 576 wild-type. Survival was monitored for 35 days after rechallenge. A delay in the expected median time to death was observed in all groups. A survival rate of 60% was observed for mice dosed with $10^4$ c.f.u. 576 bipD::pDM4 then rechallenged with $10^8$ c.f.u. wild-type 576 (Fig. 5); therefore, prior infection with the 576 bipD::pDM4 mutant partially protected mice from infection with the wild-type organism. Control mice (not age-matched) given $10^6$ c.f.u. wild-type 576 at the same time all died.

**Immunization of BALB/c mice with purified BipD protein does not protect against challenge with wild-type B. pseudomallei**

Groups of ten 7- to 9-week-old female BALB/c mice were immunized via the intraperitoneal route three times at 14 day intervals with $10 \mu$g purified BipD protein with Ribi adjuvant in PBS. Control mice were immunized with $10 \mu$g GST plus adjuvant or with PBS. A marked induction of BipD-specific IgG was detected by ELISA at day 54 after the first immunization. Titre units of IgG isotypes were as follows: total IgG, 1005; IgG1, 145; IgG2a, 1510; IgG2b, 845; IgG3, 7. On day 55 after the first immunization, mice were challenged with $3 \times 10^4$ c.f.u. B. pseudomallei strain K96243 by the intraperitoneal route and monitored for 35 days. A slight delay in median time to death was observed in the group of mice immunized with BipD compared to the control groups (Fig. 6) (median time to death BipD, 15·75±0·74 days; GST, 14·6±0·51 days; PBS,
14.3 ± 1.55 days); however, the differences were not statistically significant. An overall survival rate of 20% was observed in the group immunized with BipD, whereas immunization with GST or PBS did not protect against lethal challenge (Fig. 6).

**DISCUSSION**

We assessed the role of components of the Bsa TTSS in *B. pseudomallei* virulence and immunity. Specifically, we assessed the role of BipD, a putative translocator protein that is required for invasion of epithelial cells, survival in murine macrophage-like cells, escape from endocytic vesicles and subsequent actin-tail formation (Stevens et al., 2002, 2003). We also investigated the effect of mutation of genes encoding a type III secreted guanine nucleotide exchange factor that facilitates invasion (BopE; Stevens et al., 2003) and the putative type III secreted effector proteins BopA and BopB.

A *B. pseudomallei* strain 576 *bipD* mutant was calculated to have a MLD in BALB/c mice of $1.73 \times 10^5$ c.f.u. compared to 80 c.f.u. for the parent strain (a greater than 2160-fold reduction in MLD). Significant attenuation was also observed following administration of the *bipD* mutant to BALB/c mice via the intranasal route, which is likely to represent a more natural route of infection. Mutation of *bipD* had the same effect on virulence following intranasal inoculation when a different *B. pseudomallei* strain was used. This suggests that the attenuation of the 576 *bipD*::pDM4 mutant is unlikely to be explained by second-site mutations in the genome of the mutant strain. Furthermore, a *bsaZ* mutant of strain 10276 was attenuated to the same degree as the *bipD* mutant following intranasal infection; this confirms that the *bsa*-encoded Inv/Mxi-Spa-like TTSS plays an important role in the pathogenesis of melioidosis in mice. Our data are in agreement with the finding that structural components of the *B. mallei* strain ATCC 23344 Bsa apparatus are required for full virulence in rodents (Ulrich & DeShazer, 2004).

Splenomegaly and abscess formation were less pronounced in the liver and spleen of mice infected with the *B. pseudomallei* *bipD* mutant on days 1, 3, 5 and 7 post-infection, compared to mice infected with the wild-type strain. This is consistent with the reduced replication rate of the *bipD* mutant in the early phase of infection and the observations of Ulrich & DeShazer (2004). It is noteworthy that considerable animal-to-animal variation in bacterial load was detected with apparent clearance of the *bipD* mutant in some, but not all, animals. This reinforces the importance of examining groups of mice rather than single animals at each time interval, and it may explain why only some mice later succumbed to infection with the *bipD* mutant strain.

Mice that died or reached a humane end-point following infection with the *bipD* mutant contained only mutant bacteria upon post-mortem examination, indicating that the insertion was stable in vivo and that the mice succumbed to the overall burden of the mutant strain and not to a revertant. Attenuation of the *bipD* mutant was not complete, and was significantly less than that caused by mutation of genes required for capsular polysaccharide or branched-chain amino acid biosynthesis (Atkins et al., 2002a, b). Nevertheless, mutation of *bipD* caused a significant delay in the median time to death compared to the wild-type in C57BL/6 IL-12 p40−/− mice, which are highly susceptible to *B. pseudomallei* infection.

*B. pseudomallei* *bopE* mutants were not significantly attenuated in BALB/c mice compared to the wild-type following either intraperitoneal or intranasal inoculation. In contrast, mutation of the putative *bsa*-encoded effectors BopA and BopB caused a statistically significant increase in median time to death in BALB/c mice following intraperitoneal inoculation, at least when compared to the *bopE* mutant. BopA is a homologue of *Shigella* IscB, which is required for cell-to-cell spread of *Shigella* (Allaoui et al., 1992). *B. pseudomallei* is also capable of cell-to-cell spread and cell fusion (Kespichayawattana et al., 2000); the role of BopA in these processes is the subject of investigation in our laboratory. BopB contains a motif shared with the catalytic domains of numerous phosphatases and potentially may subvert or inhibit eukaryotic cell signalling pathways. The finding that mutations affecting known or putative effector proteins have more subtle effects on *B. pseudomallei* virulence than disruption of the translocator BipD implies that they may act in concert to influence the outcome of infection, as is the case with *Salmonella* type III secreted effector proteins (Wallis & Galyov, 2000; Zhang et al., 2003). We cannot preclude the possibility that pDM4 insertions in *bopA* and *bopB* may have disrupted the expression of nearby genes. However the observation that the *bopA* and *bopB* mutants exhibit normal actin tail formation in host cells implies that the in vivo phenotype is unlikely to be the result of indirect effects on the function of the Bsa type III secretion apparatus, since this is required for endosome escape (Stevens et al., 2002).

Mice infected with the *B. pseudomallei* *bipD* mutant were
partially protected against a challenge with the wild-type organism and the nature and efficacy of antigen-specific humoral and cell-mediated immune responses elicited by the *B. pseudomallei* *bipD* mutant requires further study. Protection conferred by the *bipD* mutant was not complete and may be influenced by the activation of innate immunity by persistent organisms at the time of rechallenge.

Immunization with purified *BipD* did not confer significant protection against *B. pseudomallei* infection. Coadministration of *BipD* with purified *BipC* did not improve protection, and *BipB* alone was not protective (J. Hill, M. W. Wood & E. E. Galyov, unpublished observations). The V-antigen proteins involved in translocation of type III secreted effector proteins in *Yersinia* and *Pseudomonas* have proven to be effective protective antigens (Leary et al., 1995; Sawa et al., 1999). It remains to be determined if other components of the *bsa*-encoded TTSS can induce protective immune responses against *B. pseudomallei*.

We have demonstrated that the *Bsa* type III protein secretion is required for full virulence of *B. pseudomallei* in mice. *B. pseudomallei* contains two other putative type III secretion loci, which are similar to the *hrp* gene cluster of *Ralstonia solanacearum* and TTSS loci in other plant pathogens (Winstanley et al., 1999; Rainbow et al., 2002); the role played by these systems in virulence awaits investigation. A mutation in the plant pathogen-like type III secretion apparatus of *Burkholderia cepacia* genovar III was recently reported to be attenuating in a murine model of respiratory infection (Tomich et al., 2003). Thus TTSSs appear to play key roles in the pathogenesis of *Burkholderia* infections in animals.

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