In vivo expression technology identifies a type VI secretion system locus in *Burkholderia pseudomallei* that is induced upon invasion of macrophages

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The Gram-negative proteobacterium *Burkholderia pseudomallei* can survive and multiply within a variety of eukaryotic cells, including macrophages. This property is believed to be important for its ability to cause the disease melioidosis in a wide range of animal species, including humans. To identify determinants that are important for the ability of *B. pseudomallei* to survive within macrophages, *in vivo* expression technology (IVET) was employed. Several putative macrophage-inducible genes were identified that are likely to contribute to the virulence of *B. pseudomallei*, including three genes (*tssH-5*, *tssI-5* and *tssM-5*) located within the same type VI secretion system cluster (*tss-5*), *mnhH*, encoding a natural resistance-associated macrophage protein (NRAMP)-like manganese ion transporter, and a haem acquisition gene, *bhuT*. The macrophage-inducibility of the *tss-5* gene cluster was confirmed by reporter gene analysis. Construction of *tssH-5* and *bhuT* null mutants indicated that expression of the *tss-5* unit and the *bhu* operon were not required for intramacrophage survival. A further five *tss* units were identified within the *B. pseudomallei* genome that, together with *tss-5*, account for approximately 2.3 % of the total genome size. The presence of six type VI secretion systems in this organism is likely to be an important factor in making this bacterium such a versatile pathogen.

INTRODUCTION

Melioidosis is the name given to any infection caused by *Burkholderia pseudomallei*, a saprophytic, Gram-negative bacillus found in wet soil and pooled water, particularly in south-east Asia and northern Australia (White, 2003). It is principally acquired following inoculation of lesions in the skin by contaminated soil and water, with the highest incidence of the disease occurring during the rainy and monsoon seasons (Dance, 1991; White, 2003). Another important route of infection is inhalation of contaminated particles. The clinical spectrum of melioidosis ranges from an acute fulminant septicaemia to chronic localized infections, often affecting the lung, and is usually

characterized by abscess formation. No vaccines are available, and antibiotic therapy is problematic due to the intrinsic high resistance of *B. pseudomallei* to many antibiotics. The overall mortality of melioidosis patients is 50 % (White, 2003).

Certain features of melioidosis suggest that *B. pseudomallei* is a facultative intracellular pathogen. These include the occurrence of long periods of latency (a recent case report suggests this can be as long as 62 years), relapses due to recrudescence of a persistent primary infection, and the activation of a cellular immune response during melioidosis (Chaowagul *et al.*, 1993; Ngaay *et al.*, 2005). Consistent with this, *B. pseudomallei* has been shown to survive and multiply within non-phagocytic cells, macrophages and free-living amoebae (Pruksachartvuthi *et al.*, 1990; Jones *et al.*, 1996; Inglis *et al.*, 2000). Once it has entered the intracellular compartment, *B. pseudomallei* is able to escape from endocytic vacuoles and move within the cytoplasm and enter neighbouring cells by inducing actin rearrangement, leading to the formation of actin tails and membrane protrusions (Kespichayawattana *et al.*, 2000; Breitbach *et al.*, 2003; Stevens *et al.*, 2005). For many bacterial pathogens, entry and survival within eukaryotic cells is dependent on a functional type III or type IV secretion system (T3SS or T4SS) (Cornelis, 2006;

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Abbreviations: EDDHA, ethylenediamine-di-(o-hydroxyphenylacetic) acid; FHA, forkhead-associated; IVET, *in vivo* expression technology; NRAMP, natural resistance-associated macrophage protein; NTD, N-terminal domain; T3SS, type III secretion system; T4SS, type IV secretion system; T6SS, type VI secretion system.

Supplementary material showing the organisation of *tss* units within proteobacterial genomes, the core components of the *tss* units, the *tss*-associated genes and a standardized nomenclature for T6SS is available with the online version of this paper.

Christie *et al.*, 2005; Segal *et al.*, 2005). The *B. pseudomallei* genome has the potential to encode three T3SSs (T3SS-1 to T3SS-3) (Attree & Attree, 2001; Rainbow *et al.*, 2002). Inactivation of components of T3SS-3 (also known as Bsa or the animal pathogen-like T3SS), which shares homology with the *Salmonella enterica* serovar Typhimurium Inv/Spa/Prg T3SS, results in impaired invasion and survival within eukaryotic cells, inability to escape from endocytic vacuoles, and failure to produce membrane protrusions and actin tails (Stevens *et al.*, 2002, 2003). Another gene, *bimA*, has recently been shown to be required for intracellular actin-based motility of *B. pseudomallei* (Stevens *et al.*, 2005). T3SS-3 is also required for full virulence of the Mallei clone in BALB/c mice and Syrian hamsters, and for intracellular survival in the J774.2 macrophage-like cell line (Ulrich & DeShazer, 2004; Ribot & Ulrich, 2006).

Several approaches have been used to identify *in vivo*-induced genes in bacterial pathogens, including signature-tagged mutagenesis (STM) and *in vivo* expression technology (IVET) (Angelichio & Camilli, 2002; Autret & Charbit, 2005). IVET allows for the study of bacterial responses to the host environment *in situ*, using a gene expression scheme for a selection of genes that are transcriptionally induced during infection. In a variation of the original approach, which avoids the requirement for specific auxotrophic strains, co-integrate strains harbouring fusions to the chloramphenicol acetyltransferase (*cat*) gene are challenged with antibiotic in the infection model (Mahan *et al.*, 1995; Young & Miller, 1997). The latter approach also proved useful for identifying bacterial genes expressed within cultured macrophages, relying on the fact that chloramphenicol can penetrate mammalian cells (Mahan *et al.*, 1995). Coupled with the recent determination of the genome sequence of *B. pseudomallei* strain K96243 (Holden *et al.*, 2004), these techniques provide powerful tools for gaining an insight into the virulence mechanisms of this bacterium.

Apart from the role of the T3SS-3, virtually nothing is known about the molecular mechanisms that allow *B. pseudomallei* to survive intracellularly. The aim of this study was to use an IVET-based approach to identify genes which may contribute to the ability of this organism to survive intracellularly. Our results indicate that at least one type VI secretion system (T6SS) gene cluster, as well as genes for at least two different metal ion acquisition systems, are induced following uptake by macrophages.

METHODS

Bacterial strains, media and growth conditions. *B. pseudomallei* 10274 was isolated from the blood of a fatal human case of melioidosis and was obtained from the NCTC, London, UK. *E. coli* CC118(λ pir) (Herrero *et al.*, 1990) was used for maintaining mobilizable R6K-derived replicons such as pGSTp, pSHAFT, pZINT2 and derivatives thereof. *E. coli* BW19851 (Metcalf *et al.*, 1994) was used for mobilization of R6K-derived plasmids into *B.*

pseudomallei. In general, *E. coli* strains were grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989), and *B. pseudomallei* was grown in brain heart infusion broth (BHIB). M9 minimal salts agar, when required, was made up according to Clowes & Hayes (1968). Bacteria were routinely grown at 37 °C. Antibiotics were incorporated into LB agar except for trimethoprim, which was incorporated into Iso-Sensitest (IST) agar (Oxoid). Antibiotic concentrations used were 100 µg ampicillin ml⁻¹, 25 µg chloramphenicol ml⁻¹, 25 µg trimethoprim ml⁻¹ and 25 µg gentamicin ml⁻¹ (unless indicated otherwise).

Determination of the minimum inhibitory concentration. The minimum inhibitory concentration (MIC) for the antibiotics amikacin, gentamicin, kanamycin, netilmicin, streptomycin, teicoplanin and vancomycin was determined by a double-dilution method in solid medium. The antibiotic concentration ranged from 1 µg ml⁻¹ to 256 µg ml⁻¹ for all the antibiotics and the MIC was recorded as the minimum antibiotic concentration that killed all the bacteria on the agar plate.

Invasion assay. RAW264.7 (American Type Culture Collection) cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco), containing 10 % fetal calf serum and 1 mM sodium pyruvate, in a 37 °C incubator with 5 % CO₂. The day before the invasion assay was carried out, cells were seeded in 24-well tissue culture plates to 1 × 10⁵ or 1 × 10⁶ cells per well. *B. pseudomallei* was grown overnight at 37 °C statically in 10 ml BHIB. The culture was then diluted 1:20 in 10 ml BHIB and grown for 4 h at 37 °C. The invasion assay was performed according to Elsinghorst (1994) with slight modifications. Bacteria from the 4 h broth culture were centrifuged at 5000 g for 10 min and resuspended in 1 ml PBS. The bacteria were then centrifuged and resuspended in 1 ml DMEM. The monolayers were infected with 25 µl of the bacterial suspension (~10⁷ cells) and incubated for 2 h at 37 °C with 5 % CO₂. The monolayers were washed with DMEM and then maintained in 1.5 ml DMEM containing both amikacin and kanamycin (500 µg ml⁻¹ each) for an additional 2 h to kill any extracellular bacteria. The monolayers were then lysed with 1 % saponin to release the intracellular bacteria. The initial inoculum and intracellular bacteria were quantified by plating serial dilutions onto blood agar plates.

Intracellular growth assays. Intracellular growth assays were carried out as described above for invasion assays, except that after killing the extracellular bacteria with amikacin and kanamycin, the monolayers were washed with PBS and then incubated overnight with 1.5 ml DMEM containing ceftazidime (200 µg ml⁻¹). RAW264.7 cells were lysed and the recovered bacteria quantified 4 and 24 h after infection of the monolayers.

Plasmid constructions. The IVET vector, pGSTp (Shalom *et al.*, 2000), contains a promoterless chloramphenicol resistance gene (*cat*) and a trimethoprim resistance (Tp^R) gene as a marker for selection in *B. pseudomallei*. The integrative *lacZ* transcription fusion vector, pZINT2 [formerly pUTmini-Tn5TlacZYAΔBAK (Lowe *et al.*, 2001)], was constructed from pUTmini-Tn5TlacZYA (A. H. Asghar and others, unpublished) by deletion of the transposase gene and I end of mini-Tn5Tp (Lowe, 2001). Details of its construction will be reported elsewhere. pGS501 was constructed by inserting an approximately 600 bp *XhoI*–*XbaI* DNA fragment, containing the 3' end of *tssH*-5 and 5' end of *tssI*-5, between the *Sall* and *XbaI* sites located upstream of the promoterless *lacZ* gene in pZINT2.

To construct pSHAFT, the transposase gene of pUTmini-Tn5Cm (de Lorenzo *et al.*, 1990) and part of the I end repeat sequence adjacent to the chloramphenicol resistance interposon was removed by digestion with *Bgl*II, followed by self-ligation of the 7.5 kb vector fragment. The resultant plasmid has unique sites for *Bgl*II, *Not*I, *Sac*II and *Sall* that

permit insertion of genes to be targeted for inactivation on the chromosome. pSHAFT encodes resistance to ampicillin as well as chloramphenicol. pUT41Tp and pUT49Tp are derivatives of pSHAFT that were used to create chromosomal *bhuT* and *tssH-5* mutants, respectively. The progenitor of pUT41Tp, pVPCR41, was generated by Vectorette PCR (Sigma-Genosys; see below) from co-integrate strain GS41, and consists of an 850 bp *NarI* fragment harbouring the 3' end of *bhuS* and 5' end of *bhuT* cloned into the *EcoRI* site of pUC19. The Tp^R cassette from p34E-Tp (DeShazer & Woods, 1996) was then cloned into the unique *PmlI* site of *bhuT*, giving rise to pVPCR41Tp. The '*bhuS-bhuT*': Tp^R fragment was then transferred from pVPCR41Tp as a 1.5 kb Klenow-filled *EcoRI*-*XbaI* fragment into the Klenow-filled *BglII* site of pSHAFT, resulting in pUT41Tp. The progenitor of pUT49Tp, pVPCR49, was generated by Vectorette PCR from co-integrate strain GS49, and harbours a 1034 bp DNA fragment encoding the C-terminal region of TssH-5 and N-terminal region of TssI-5. The Tp^R cassette of p34E-Tp was then cloned into the *XhoI* site located 404 bp upstream from the predicted *tssH-5* translational stop codon, giving rise to pVPCR49Tp. The 1.7 kb '*tssH-5*': Tp^R -*tssI-5*' fragment was then transferred as a Klenow-filled *EcoRI*-*XbaI* fragment to the filled *BglII* site of pSHAFT, resulting in pUT49Tp.

Construction of a pGSTp co-integrate library in *B. pseudomallei*. *B. pseudomallei* 10274 genomic DNA was partially digested with *Sau3AI*, following which fragments of between 0.5 and 1.0 kb in length were purified from an agarose gel and inserted into the *BglII* site of pGSTp. The resultant plasmid library was used to transform *E. coli* CC118(λ pir). Approximately 3.4×10^4 transformants were pooled, amplified, and total plasmid DNA was isolated. A sample of the plasmid library was used to transform *E. coli* BW19851 and thereafter introduced into *B. pseudomallei* 10274 by conjugal transfer as described previously (Herrero *et al.*, 1990; de Lorenzo & Timmis, 1994). *B. pseudomallei* exconjugants, obtained at a frequency of 1.2×10^{-6} per recipient, were selected on IST agar containing trimethoprim and gentamicin and pooled in BHIB.

IVET selection in macrophages. One hundred microlitres of the co-integrate pool ($\sim 2 \times 10^9$ cells) were grown overnight. RAW264.7 cells were seeded at 1×10^5 cells per well and infected at an m.o.i. of 100. After killing extracellular bacteria with amikacin and kanamycin, the infected macrophages were treated with chloramphenicol ($100 \mu\text{g ml}^{-1}$) overnight or left untreated. The following day, the macrophages were lysed and the co-integrate strains were recovered on IST agar containing trimethoprim. The recovered co-integrate strains were pooled together separately and grown overnight for another round of macrophage infection and selection; this was repeated for a total of four rounds.

Identification of macrophage-induced genes. The presence of pGSTp in the genome of co-integrate strains was confirmed by Southern blotting (Sambrook *et al.*, 1989) using the *dfr* (Tp^R) gene as a probe following digestion of genomic DNA with *PstI* (which cuts within the *bla* gene of pGSTp). To clone regions of genomic DNA flanking the inserted plasmid, Vectorette PCR was performed according to the manufacturer's instructions (Sigma-Genosys). Genomic DNA from the co-integrate strains was digested with *EcoRI*, *NarI*, or *EcoRI* and *Clal* in combination. Following this, compatible linkers (Vectorettes) were ligated to the ends of the chromosomal DNA fragments and PCR was then performed using a primer homologous to the 5' end of *cat* gene in pGSTp (Cat-IPEco, 5'-CCCGAATTGCACTGAAATGCCTC-3') and a primer homologous to the linker sequence. The resultant PCR products were ligated into pUC19 and their nucleotide sequences were determined.

Construction of a *tssH-5-lacZ* fusion strain and measurement of β -galactosidase activity in macrophages. pGS501 was

mobilized into *B. pseudomallei* as described previously (Herrero *et al.*, 1990; de Lorenzo & Timmis, 1994) and exconjugants were selected on IST agar containing trimethoprim and gentamicin. Integration of the plasmid into the genome at the expected site by a single crossover was confirmed by Southern blotting. One such co-integrate strain, GS549, was added to tissue culture medium ($\sim 10^7$ bacteria per well) either containing or not containing RAW264.7 cells ($\sim 10^5$ per well). Following incubation for 2 h at 37 °C, non-internalized bacteria were killed by treatment with amikacin and kanamycin ($500 \mu\text{g ml}^{-1}$ each) for a further 2 h, following which bacteria were recovered by lysis of the RAW264.7 cells with 0.5 ml 1% saponin. The recovered bacteria (200 μl) were mixed with 800 μl Z buffer (Miller, 1972) and then permeabilized with 30 μl chloroform and 30 μl 0.1% SDS. The assay was carried out at 30 °C according to Miller (1972). β -Galactosidase activities (expressed in arbitrary units, Au) were determined by normalizing the rate of ONPG hydrolysis to the number of c.f.u. used in the assay. Control bacteria (which were not exposed to macrophages or antibiotics) were treated in an otherwise identical fashion.

Construction of *B. pseudomallei tssH-5* and *bhuT* null mutants. pUT41Tp and pUT49Tp were mobilized into *B. pseudomallei* and exconjugants were selected on IST agar containing trimethoprim and gentamicin. *B. pseudomallei* exconjugants in which the introduced *bhuT* and *tssH-5* null alleles had been transferred to the chromosome by a double-crossover event arose at a frequency of 2–8%, and were identified by virtue of their sensitivity to chloramphenicol. Transfer of the null *bhuT* and *tssH-5* alleles to the correct genomic locus was confirmed by Southern blotting (Sambrook *et al.*, 1989) and the resultant mutants were designated GS241 and GS249, respectively.

Haem utilization assay. *B. pseudomallei* was assessed for its ability to utilize haemin as an iron source by a plate bioassay. Overnight cultures were diluted 100-fold in PBS and 100 μl of the diluted culture was mixed with 5 ml soft LB agar (0.65%) and poured onto LB agar plates containing the iron chelator ethylenediamine-di-(*o*-hydroxyphenylacetic) acid (EDDHA, 200 μM). Once the soft agar had solidified, 10 mm diameter sterile filter paper discs (Whatman), spotted with 10 μl of a $10 \mu\text{g ml}^{-1}$ solution of haemin, were placed onto the surface of the plate, which was then incubated overnight at 37 °C. Plates were examined for zones of growth around the haemin-impregnated filter discs.

Bacterial sensitivity to hydrogen peroxide and low pH. Sensitivity to hydrogen peroxide was determined by a plate assay method. Overnight cultures were diluted tenfold in BHIB and 1–2 ml of the diluted culture was used to flood the entire surface of the LB agar plate; any excess liquid culture on the plate was removed. Once the agar surface was dry, 10 mm sterile filter paper discs (Whatman), spotted with 10 μl of 1 M hydrogen peroxide, were placed onto the agar surface. Following overnight incubation at 37 °C, the diameter of the zones of clearing surrounding the discs was measured. To assess the sensitivity of *B. pseudomallei* to low pH, approximately 10^{10} bacteria were inoculated into BHI broth that had been adjusted to pH 4.0 and incubated at 37 °C for 24 h. The number of viable bacteria present was determined at 6 h intervals by performing plate counts.

RESULTS

Intracellular survival of *B. pseudomallei* in a macrophage-like cell line

The MICs of a range of β -lactam and aminoglycoside antibiotics for *B. pseudomallei* 10274 were determined in

order to identify those most appropriate for use in invasion assays. These two classes of antibiotics either penetrate phagocytes poorly or show little or no activity against intracellular bacteria (Pascual, 1995). From the results obtained, amikacin proved to be the most effective single antibiotic for killing extracellular *B. pseudomallei* (MIC $64 \mu\text{g ml}^{-1}$). Invasion assays were then performed to assess the invasive capability of *B. pseudomallei* strain 10274 in the macrophage-like cell line, RAW264.7. A number of parameters were varied to optimize the assay, such as the time allowed for internalization, the nature and concentration of the antibiotics used to kill non-internalized bacteria, and the length of time of exposure to the antibiotic. It was found that exposure to amikacin in combination with kanamycin (both at $500 \mu\text{g ml}^{-1}$) for 2 h, following 2 h to allow for infection, was most effective. Under these conditions, the mean number of intracellular bacteria was approximately 3×10^5 per well. After overnight incubation (a further 15 h), the mean number of intracellular bacteria was 3.7×10^4 per well. Although there was a log unit decrease in the number of viable intracellular bacteria over this period, the number of bacteria remaining viable at this time point (19 h) was more than adequate to apply an IVET approach to this system.

IVET selection of macrophage-induced genes in *B. pseudomallei*

The IVET system used in this study relied on the ability of chloramphenicol to penetrate eukaryotic cells and kill intracellular bacteria containing a chromosomally integrated *cat* gene that is only weakly expressed. To ascertain the optimum concentration of chloramphenicol to be used, intracellular growth assays were performed in RAW264.7 cells. After overnight incubation, the mean number of intracellular bacteria recovered from the infected monolayers in the absence of exposure to chloramphenicol was $1.8 \pm 0.6 \times 10^4$ per well, whereas monolayers cultured in the presence of chloramphenicol at concentrations of $25 \mu\text{g ml}^{-1}$ and $100 \mu\text{g ml}^{-1}$ contained $9.9 \pm 1.7 \times 10^2$ and $3.2 \pm 0.8 \times 10^2$ c.f.u. per well, respectively. As chloramphenicol was slightly more effective at $100 \mu\text{g ml}^{-1}$, this concentration was used for the IVET selection.

A *B. pseudomallei* genomic DNA library was constructed in the IVET vector, pGSTp, and mobilized into *B. pseudomallei* 10274 to generate a library of co-integrate strains in which plasmid recombinants had integrated by homologous recombination into the *B. pseudomallei* genome at different loci. Strains containing fusions of the *cat* gene to genes that are transcriptionally active within macrophages were selected by repeatedly passaging the *B. pseudomallei* co-integrate pool through RAW264.7 cells, during which time the intracellular bacteria were subjected to a chloramphenicol challenge. After four rounds of selection, there was no further reduction in the number of co-integrate strains recovered from the chloramphenicol-treated macrophages compared to the untreated macrophages.

Of a total of 4115 co-integrate strains that survived chloramphenicol challenge, 420 (i.e. 10.2 %) were found to be sensitive to $10 \mu\text{g ml}^{-1}$ chloramphenicol (i.e. just above the MIC) when plated on LB agar. These strains were expected to contain fusions of *cat* to genes which are active within macrophages but which are not necessary for growth on nutrient-rich medium. In comparison, 75 % of co-integrate strains from the pre-selection pool were found to be chloramphenicol-sensitive on laboratory media. We presume that this change in frequency is due to the elimination of co-integrate strains in which the *cat* gene remains quiescent during passage through macrophages. To eliminate co-integrate strains in which *cat* is fused to genes required for the biosynthesis of essential metabolites or the production of ion transport systems, the chloramphenicol-sensitive co-integrate strains were patched onto M9-glucose minimal agar containing $50 \mu\text{g}$ chloramphenicol ml^{-1} (i.e. just above the MIC on this medium). Of the 420 co-integrate strains containing macrophage-inducible *cat* fusions, 110 did not grow on nutrient-poor medium containing chloramphenicol, suggesting that they contained fusions of *cat* to genes which are specifically induced following uptake by macrophages.

Identification of a macrophage-induced T6SS in *B. pseudomallei*

Southern hybridization analysis suggested that, within the pool of 110 macrophage-inducible *cat* fusion strains, pGSTp had integrated at 15 different genomic loci (results not shown). The *cat* gene plus flanking genomic DNA from several different co-integrate strains was cloned into pUC19 for DNA sequence analysis. The genomic insertion sites of pGSTp in the selected co-integrates are shown in Fig. 1. DNA sequence analysis revealed that in three of the co-integrate strains, GS9, GS49 and GS66, pGSTp had integrated within ORFs which we have termed *tssM*-5, *tssI*-5 and *tssH*-5, respectively, that are located within 14 kb of each other on the *B. pseudomallei* small chromosome (chromosome 2) (Figs 1 and 2). In the recently published *B. pseudomallei* genome sequence, *tssH*-5, *tssI*-5 and *tssM*-5 have been assigned locus tags BPSS1502, BPSS1503 and BPSS1511, respectively (Holden *et al.*, 2004). The translated products of these ORFs exhibit strong homology to ClpV (*tssH*-5), Vgr-like proteins (*tssI*-5) and IcmF-family proteins (*tssM*-5) (Schlieker *et al.*, 2005; Wang *et al.*, 1998; Das & Chaudhuri, 2003). These genes form part of a cluster of 19 ORFs (locus tags BPSS1493–BPSS1511; *tssA*-5 to *tssM*-5 and *tagAB*-5 to *tagD*-5), spanning 28.6 kb, which appear to be organized into two or three transcriptional units based on gene orientation and the tight spacing of adjacent ORFs (almost all of the ORFs are separated by a distance of <30 bp, and in many cases the stop and start codons of adjacent ORFs overlap) (Fig. 2). At only two locations within this cluster is the intergenic region large enough to accommodate a promoter [i.e. between the divergently arranged *regA* and *tssB*-5 genes (259 bp), and between *tssC*-5 and *tssD*-5 (219 bp)]. Two regulatory genes occur within the

Fig. 1. Location of pGSTp insertions within the *B. pseudomallei* genome. Sites of pGSTp insertion in each co-integrate strain are shown by black triangles, with the orientation of the *cat* reporter gene indicated by an arrow. The region separating the *tssI-5* and *tssM-5* genes (7.8 kb) contains orthologues of *tssJ-tssL* and *tagAB-tagD* (see Fig. 2). Multiple tandem-repeat sequences flanking *bhuU* are indicated by an upper case 'R'. The Fur box overlapping the *bhu* operon promoter is denoted by an upper case 'F'. The locus tags of the genes shown are, from top to bottom, BPSS1502 (*tssH-5*) to BPSS1511 (*tssM-5*), BPSS0246 (*orfW*) to BPSS0239 (*orfY*), BPSL1558 (*potI*) to BPSL1552 (*ompW*), BPSLr06 (16S rRNA) to BPSL3036 (*orfI*) and BPSL3068 (*orfA*) to BPSL3077 (*orfG*). Predicted functions of the gene products are: *bhu*, haem uptake; *tss*, type VI secretion; *fadD*, fatty acid-CoA ligase; *hemA*, glutamyl-tRNA reductase; *hemK*, polypeptide chain release factor methylase; *mntH*, magnesium ion transport; *ompW*, outer-membrane protein; *orfA*, hydrolase; *orfB*, isochorismatase; *orfC*, aminopeptidase; *orfD*, membrane protein; *orfE*, glutaredoxin-related protein; *orfF*, octaprenyl hydroxybenzoate carboxy-lyase; *orfG*, catechol dioxigenase; *orfH*, oxidoreductase/dehydrogenase; *orfI*, outer-membrane porin; *orfW*, LysR regulator; *orfX*, hypothetical protein; *orfY*, CH membrane protein; *orfZ*, iron-sulphur protein; *pot*, polyamine (i.e. putrescine/spermidine) transport system; *prfA*, peptide chain release factor; 5S, 5S rRNA; 16S, 16S rRNA.

cluster, *regA* and *regB*, which are predicted to encode a two-component sensor kinase-response regulator system.

Potential orthologues of the 13 *tss* genes are found clustered in other members of the phylum *Proteobacteria* that either are pathogenic to animals or plants (i.e. *Salmonella enterica*, *Vibrio cholerae*, *Yersinia paratuberculosis*, *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*, *Ralstonia solanacearum*, and pathogenic strains of *Escherichia coli*) or form intimate associations with eukaryotic cells (i.e. *Rhizobium leguminosarum*, *Agrobacterium tumefaciens* and *Vibrio*

fischeri). Some examples of *tss* clusters are shown in Supplementary Figure S1 (available with the online version of this paper). It has recently been shown that *tss*-like clusters [formerly termed IAHP (IcmF-associated homologous proteins) clusters (Das & Chaudhuri, 2003)] encode a new type of secretion system, termed the type VI secretion system (T6SS) (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006).

Identification of other macrophage-induced genes in *B. pseudomallei*

In co-integrate strain GS41, the pGSTp derivative had integrated into a gene encoding a putative orthologue of the periplasmic haem-binding protein, HemT/HmuT, of *Yersinia enterocolitica*/*Yersinia pestis*. This gene, named *bhuT* (for *Burkholderia* *haem* *uptake*), was found to form part of a putative transcriptional unit organized as *bhuRSTUV* (BPSS0244–BPSS0240) (Fig. 1). Orthologues of these genes encode haem uptake systems in other Gram-negative bacteria (Wandersman & Stojiljkovic, 2000; Genco & Dixon, 2001). Orthologues of BhuR, such as HemR/HmuR, and PhuR of *P. aeruginosa*, constitute specific TonB-dependent outer-membrane receptors for haem. The BhuU and BhuV orthologues, HemU/HmuU and HemV/HmuV, form the permease and ATPase components, respectively, of the cytoplasmic membrane haem transporter. The *Shigella* BhuS orthologue, ShuS, has been suggested to act as a shuttle protein that potentiates the utilization or degradation of haem, depending upon the prevailing conditions (Wyckoff *et al.*, 2005). Upstream of *bhuR* is the sequence 5'-TTGAATN₁₇TACAAT-3', which is similar to the consensus promoter sequence recognized by the major form of RNA polymerase. Overlapping the putative *bhuR* promoter (on the opposite strand) is the sequence 5'-TGTAATTGAGAATCATTCGCA-3', which displays a 15/21 match (underlined bases) to the consensus binding site for the global iron regulator, Fur (Andrews *et al.*, 2003).

In co-integrate strains GS67, GS120 and GS127, the pGSTp derivative had integrated into intergenic regions at three different loci such that the *cat* reporter gene was inserted in the same orientation as the downstream ORF (Fig. 1). In GS67, the downstream ORF (locus tag BPSL1554) encoded a protein with high similarity to MntH. MntH is a divalent metal ion transporter with a preference for manganese and, to a lesser extent, iron, and belongs to the eukaryotic NRAMP (natural resistance-associated macrophage protein) family of proton-coupled metal ion transporters (Forbes & Gros, 2001). The *mntH* gene of *B. pseudomallei* is located 420 bp upstream of a gene of same polarity, which is predicted to encode a small (67 aa) iron-sulphur protein. Both genes are flanked by ORFs which are transcribed in the opposite direction, suggesting that *mntH* may be part of a mono- or bi-cistronic unit. In GS120, pGSTp had inserted upstream of an ORF (BPSL3038) encoding a protein with high homology to a class of anaerobic dehydrogenases that includes YoaE (formate dehydrogenase) of *Bacillus subtilis*. In GS127, pGSTp was located

Fig. 2. The *B. pseudomallei* *tss-5* unit. The *tss-5* unit is shown in its genomic context. The *tss* genes (which form a conserved core in all *tss* clusters) are uniformly coloured, whereas the *tag* genes are hatched. Non-*tss*-associated genes are shown in grey. Regulatory genes, *regA* and *regB*, which may play a role in *tss* gene expression, are shown in white. *tssA-5* through to *tssM-5* correspond to BPSS1493–BPSS1511. The *tagAB-5* gene product has an N-terminal domain (NTD) that is homologous to TagA orthologues (blue hatching), and a C-terminal pentapeptide repeat domain similar to TagB (pink hatching). The ORF located between *bimA* (BPSS1492) and *tssA-5* encodes a predicted lipoprotein (www.cbs.dtu.dk/services/LipoP) but has not been assigned a locus tag in the annotated *B. pseudomallei* sequence, although an ORF (BMAA0748) of almost identical sequence has been assigned to the corresponding region of the *B. mallei* genome (Nierman *et al.*, 2004). The product of BPSS1512 exhibits homology to ubiquitin carboxyl-terminal hydrolase 1. *folE* (BPSS1514) encodes a putative GTP cyclohydrolase I orthologue. Only the *tss-5*-proximal part of the T3SS-3 (Bsa) cluster is shown (BPSS1520–BPSS1526). The predicted Rho-independent transcription terminator located downstream of the lipoprotein gene is depicted as a ball on a stick. Tnp, transposase.

upstream of *hema* (BPSL3072), encoding glutamyl-tRNA reductase, an enzyme that catalyses the first step in the biosynthesis of tetrapyrroles such as haem.

Stimulation of *tss-5* unit transcription by macrophages

To confirm that genes within the *tss-5* unit are upregulated within macrophages, strain GS549 was constructed, containing a single-copy *tssI-lacZ* fusion integrated at the *tss-5* locus of chromosome 2. Invasion assays were performed on this strain, and the β -galactosidase activity was compared with the activity measured in bacteria treated under the same conditions in the absence of macrophages. In GS549, there was a 12-fold increase in β -galactosidase activity when the bacteria were co-cultured with macrophages (i.e. 207 ± 40 Au) in comparison to the activity in bacteria growing in the absence of macrophages (16 ± 2 Au). This result demonstrates that the predicted transcriptional unit extending from *tssD-5* through to *tssM-5* is induced by the presence of, or interaction with, macrophages.

Effect of inactivation of *tssH-5* on invasion of macrophages

The role of the macrophage-inducible *tss-5* locus in invasion and intracellular survival of *B. pseudomallei* within macrophages was examined. To do this, we constructed a mobilizable suicide vector, pSHAFT, for

the specific purpose of carrying out allelic replacement in members of the genus *Burkholderia* (see Methods). A derivative of pSHAFT containing an inactivated copy of *tssH-5* was then used to construct the *B. pseudomallei* derivative GS249, in which the chromosomal *tssH-5* gene was disrupted by a trimethoprim resistance (*dfr*) cassette. Overnight (19 h) invasion assays were performed with GS249, using the RAW264.7 cell line. Our results showed that there was no significant difference between the number of wild-type and mutant *B. pseudomallei* located intracellularly (Table 1). Thus, the mutant strain is as proficient as the wild-type strain at invading and surviving within the RAW264 cell line. *clpV* orthologues such as *tssH-5* are also highly homologous to *clpB* (Schlieker *et al.*, 2005). It has been shown previously that a *Brucella suis* *clpB* mutant exhibited increased sensitivity to both acid and peroxide stress (Ekaza *et al.*, 2001). Therefore, we examined the sensitivity of the *tssH-5* mutant to low pH and to hydrogen peroxide. Our results showed that the wild-type and *tssH-5* mutant strains were equally sensitive to acid stress at pH 4.0 (Table 2) and to hydrogen peroxide (diameter of zone of killing ~ 42 mm in both cases).

Effect of inactivation of *bhuT* on haem utilization and invasion of macrophages

The role of *bhuT* in invasion and intracellular survival of *B. pseudomallei* within macrophages was examined. To do this, a mutant strain, GS241, was constructed, containing a

Table 1. Intracellular survival of *B. pseudomallei* *tssH*-5 and *bhuT* mutants

Data are expressed as means $\pm$ SD. Invasion assays were performed in triplicate.

Strain	Inoculum (c.f.u. per well)	Intracellular bacteria (c.f.u. per well)	
		4 h	19 h
NCTC 10274	$1.3 \pm 0.2 \times 10^7$	$3.1 \pm 1.1 \times 10^5$	$5.6 \pm 2.9 \times 10^3$
GS249 (<i>tssH</i> -5::dfr)	$1.1 \pm 0.1 \times 10^7$	$1.8 \pm 0.3 \times 10^5$	$7.2 \pm 3.6 \times 10^3$
GS241 (<i>bhuT</i> ::dfr)	$1.5 \pm 0.2 \times 10^7$	$7.4 \pm 0.4 \times 10^5$	$9.0 \pm 5.5 \times 10^3$

bhuT::dfr null allele. Overnight invasion assays using the RAW264.7 cell line showed that there was no significant difference between the number of wild-type and mutant *B. pseudomallei* located intracellularly (Table 1). One possible reason for the above observation is that inactivation of *bhuT* does not compromise the ability of the strain to take up haem. To determine whether this is the case, the effect of disruption of *bhuT* on haem utilization was examined using a plate assay. Our results showed that the wild-type strain formed a zone of growth around the haem-impregnated disc (diameter ~21 mm) but not around a PBS-saturated disc (results not shown), indicating that *B. pseudomallei* can utilize haem as an iron source. The *bhuT* mutant strain also formed a zone of growth of similar size around the haem-impregnated filter, suggesting that the lesion did not completely disrupt the function of the Bhu system or that *B. pseudomallei* possesses an alternative system for haem uptake.

DISCUSSION

We have used a modification of the original IVET procedure to identify macrophage-induced genes. Two of the identified macrophage-inducible genes are highly homologous to the *bhuT* and *mntH* genes that are required for haem and manganese transport in other bacteria. The divalent metal ion transporter, MntH, is important for manganese homeostasis, and it has been shown to play a role in the virulence of several pathogens, particularly in

Table 2. Effect of *tssH*-5::dfr mutation on sensitivity of *B. pseudomallei* to low pH

The data are expressed as means $\pm$ SD. Assays were performed in triplicate.

Strain	Total c.f.u. at time:		
	0 h	6 h	24 h
NCTC 10274	8.3×10^9	$4.1 \pm 0.8 \times 10^8$	$3.0 \pm 0.6 \times 10^8$
GS249 (<i>tssH</i> -5::dfr)	1.0×10^{10}	$3.2 \pm 0.8 \times 10^8$	$3.0 \pm 0.6 \times 10^8$

relation to resistance to reactive oxygen species (Zaharik & Finlay, 2004). Haem is the most abundant source of iron in animal hosts and, accordingly, haem uptake systems have proven to be important contributors to the virulence of several bacterial pathogens (Wandersman & Stojiljkovic, 2000; Genco & Dixon, 2001). For example, *Staphylococcus aureus* has been shown to prefer haem as an iron source during infection, and haem uptake is required for full virulence of uropathogenic *E. coli* (Torres *et al.*, 2001; Skaar *et al.*, 2004). The presence of a Fur box overlapping the putative promoter for *bhuR* suggests that the haem uptake system of *B. pseudomallei* is induced in response to iron starvation. Consistent with this, microarray analysis has shown that transcription of BPSS0243 (*bhuS*) and BPSS0244 (*bhuR*) is induced under iron-limiting conditions (Tuanyok *et al.*, 2005; Alice *et al.*, 2006). It has been observed previously that iron acquisition genes are required by other intracellular pathogens, such as *Legionella pneumophila*, for growth within macrophages (Pope *et al.*, 1996).

Significantly, three of the macrophage-inducible genes are located within a cluster of genes (*tss*-5) that encode a newly described secretion system in Proteobacteria, the T6SS (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). Our survey of *tss*-like clusters reveals that they contain 13 conserved genes (*tssA*–*tssM*) which we propose constitute the 'core' *tss* unit (Supplementary Table S1; M. S. Thomas, unpublished). As yet, specific functions have not been assigned to individual T6SS components. The products of *tssD* (*hcp*) and/or *tssI* (*vgr*) orthologues appear to be secreted, and they may constitute surface components of the T6SS or be part of a secreted translocon (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). In this regard, Hcp has been shown to form ring-like structures which may act as a channel through which other macromolecules pass (Mougous *et al.*, 2006). TssH (ClpV) is a member of the AAA+ superfamily of ATPases (Neuwald *et al.*, 1999), and is similar to the ClpB chaperone (Hanson & Whiteheart, 2005; Schlieker *et al.*, 2005). Recently, TssH has been hypothesized to provide the force for the translocation of exported proteins by the T6SS (Mougous *et al.*, 2006). The IcmF-like TssM orthologues contain an N-terminal domain that is similar to members of the small GTPase superfamily present in eukaryotes, and includes a Walker box A nucleotide-binding site (Sexton *et al.*, 2004; Zusman *et al.*, 2004; M. S. Thomas, unpublished). In the *L. pneumophila* T4SSB, IcmF and DotU (IcmH) are proposed to form an inner membrane complex that serves to stabilize or optimize the function of the secretion machinery (Sexton *et al.*, 2004; VanRheenen *et al.*, 2004). Due to the similarity of TssL to DotU, TssL and TssM are likely to form a similar complex.

tss units are distinguished by the presence of additional *tss*-associated genes (*tags*) which vary in number and function from one unit to another (Supplementary Figure S1, available with the online version of this paper). Tags include predicted serine/threonine protein kinases (TagE) and phosphatases (TagG), forkhead-associated (FHA)

domain proteins (TagH), and polypeptides comprising multiple pentapeptide repeats (TagAB and TagB) (Supplementary Table S2). It is possible that some or all of these proteins may be secreted by the T6SS. However, a number of Tags appear to be anchored to the cell envelope due to the presence of predicted transmembrane domains (TMDs), lipid modification sites for anchoring to the outer membrane, or MotB/OmpA-like peptidoglycan-binding domains (TagI, TagL–TagN, TagP). The latter class of proteins may play an auxiliary role in T6SS function. Due to inconsistency in the nomenclature of T6SS components, a new universal system of nomenclature is proposed (see Supplementary Table S3 for cross-referencing between previously named T6SS genes and the new nomenclature).

Although bacteria encoding T6SSs generally contain only one *tss*-like cluster, *Vibrio parahaemolyticus*, *P. aeruginosa* and *Y. pseudotuberculosis* contain two, three and four *tss* units, respectively. Interestingly, we identified a further five *tss* clusters in the *B. pseudomallei* genome, only one of which (*tss*-1) is located on the large chromosome (Fig. 3). Thus, 4.5 % of the *B. pseudomallei* small chromosome is devoted to T6SS function. This is consistent with the

observation that chromosome 2 exhibits a bias towards accessory functions that might be required for survival in various niches (Holden *et al.*, 2004). The presence of six *tss* units (*tss*-1 to *tss*-6) in the *B. pseudomallei* genome is more than in any other bacterium for which a complete genome sequence is available, and it is likely that the presence of such a large number of T6SSs (comprising ~2.3 % of its genome) increases the potential of *B. pseudomallei* to occupy a variety of niches.

There is abundant evidence that *tss* units play a role in host–pathogen interactions. For example, the *tss*-1-encoded *tssH* (*clpV*) orthologue of *P. aeruginosa* (PA0090) has been shown to be required for virulence in a rat model of chronic respiratory infection, and low-level expression of a dominant-negative *tssH* mutant blocks the invasion of HEp-2 epithelial cells by *S. enterica* serovar Typhimurium (Potvin *et al.*, 2003; Schlieker *et al.*, 2005). The *tssM* gene of the *P. aeruginosa* *tss*-1 unit has been shown to be essential for infection of a rat model of chronic respiratory infection (Potvin *et al.*, 2003), and the *tssM* orthologue of *S. enterica* sv. Typhimurium (*sciS*) has been shown to control intracellular multiplication of the

Fig. 3. The *tss* units of *B. pseudomallei*. The six *tss* units of *B. pseudomallei* strain K96243 are shown. The locus tags are given in parentheses, followed by the G + C mol% (the average G + C content of *B. pseudomallei* is 68 %). *tag* genes are indicated by diagonal hatching and labelled accordingly. Colour coding of *tss* and *tag* genes is the same as used in Fig. 2 and Supplementary Figure S1. For clarity, the *tss* prefix is omitted. The *Rhs* element present within the *tss*-2 unit is indicated by green vertical hatching. [A *tssI* (*vgr*) orthologue is not present in the *tss*-1 cluster, but *tssI* orthologues are present at other non-*tss* loci in the *B. pseudomallei* genome.]

bacterium during the later stages of infection (Parsons & Heffron, 2005). Inactivation of the *tssM* orthologue (*vasK*) of a serogroup O37 *V. cholerae* strain renders the bacterium non-virulent towards *Dictyostelium* (Pukatzki *et al.*, 2006). The same phenotype was observed for *V. cholerae* *vasA* (*tssF*) mutants, and mutants in which both copies of the *tssD* orthologue (*hcp*) were inactivated. Similarly, *Edwardsiella tarda* *evpC* (i.e. *hcp* or *tssD*) mutants were shown to be defective for intramacrophage growth and exhibited decreased virulence towards gouramis (Rao *et al.*, 2004).

Interestingly, the *B. pseudomallei* *tss-5* unit is flanked by *bimA* (BPSS1492) on one side, and the T3SS-3 locus (BPSS1520–BPSS1552) on the other (Fig. 2). T3SS-3 has been shown to be important for growth of *B. pseudomallei* within macrophage-like cells and for escape from endocytic vacuoles, and one of the associated effector proteins, BopE (BPSS1525), is required for efficient invasion of epithelial cells (Stevens *et al.*, 2002, 2003; Ribot & Ulrich, 2006; Pilatz *et al.*, 2006). T3SS-3 is also required for maximum virulence of *B. pseudomallei* in murine and hamster models of infection (Stevens *et al.*, 2004; Ulrich & DeShazer, 2004; Warawa & Woods, 2005). The *bimA* gene has recently been shown to be required for actin-based motility of *B. pseudomallei* in a macrophage-like cell line, but it is not required for Bsa-dependent escape from endocytic vesicles (Stevens *et al.*, 2005). Moreover, microarray analysis has shown that genes located upstream of *bimA* (i.e. BPSS1485, BPSS1489 and BPSS1491) are co-regulated with genes located within the T3SS-3 and *tss-5* clusters under certain conditions (Moore *et al.*, 2004). This is consistent with our observation that genes within the *tss-5* gene cluster are macrophage-inducible, and suggests that a ‘macrophage-inducible archipelago’ (MIA) of at least 76 kb in size exists within the small chromosome. The presence of a 4.5 kb region possessing a high content of A+T-rich sequences and scrambled transposase genes located between the *tss-5* and T3SS-3 units indicates that one or both of these units may have been acquired by a horizontal gene transfer event.

Pertinently, in a screen for transposon mutants of *B. pseudomallei* that exhibited a decreased ability to plaque on PtK2 cell monolayers, one of the mutants obtained carried an insertion in BPSS1509, encoding a hypothetical protein (Pilatz *et al.*, 2006). This mutant was shown to be less virulent than the wild-type strain in a mouse model of infection (Pilatz *et al.*, 2006). BPSS1509 corresponds to the *tssK* orthologue in the macrophage-inducible *tss-5* unit. Our observation that inactivation of *tssH-5* does not significantly affect the ability of *B. pseudomallei* to survive in macrophages is in agreement with the observation of Pilatz *et al.* (2006) regarding the normal growth phenotype of the *tssK-5* mutant in macrophages. Moreover, in the three co-integrate strains that we isolated in which pGSTp was integrated in the *tss-5* unit, it is likely that expression of the *tss* genes located downstream of the integrated vector were also disrupted (as the genomic fragments cloned into

pGSTp were too small to contain the predicted promoter region). Nevertheless, the co-integrate strains were still able to survive within macrophages and resist chloramphenicol challenge. One explanation for these observations is that although *tss-5* may be induced during the association of *B. pseudomallei* with different cell types, it plays a more important role in invasion of some cell types compared to others.

In conclusion, our observations suggest that, in addition to genes involved in iron and manganese homeostasis, genes required for biosynthesis of at least one T6SS are induced upon *B. pseudomallei* invasion of macrophages. Furthermore, *B. pseudomallei* contains five additional T6SSs. The identification of such a large number of T6SSs in *B. pseudomallei* opens up new avenues of investigation into the virulence mechanisms of this versatile pathogen.

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