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Characterization of *Burkholderia pseudomallei* and *Burkholderia pseudomallei*-like strains

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SUMMARY

Previous reports in the literature suggest that *Burkholderia pseudomallei* strains can be differentiated on the basis of animal virulence. Twenty environmentally and clinically derived isolates of *Burkholderia pseudomallei* were examined for the production of exoenzymes, morphological and biochemical phenotypes and virulence for Syrian golden hamsters. The partial sequence of the 16S ribosomal RNA [rRNA] genes from a number of these strains was also determined. Based upon these observations, it is suggested that highly virulent *Burkholderia pseudomallei* strains are true *Burkholderia pseudomallei* strains. The DNA sequences of the 16S rRNA genes of the true *Burkholderia pseudomallei* strains were identical to the published sequences for *Burkholderia pseudomallei* while differences were revealed between the published sequences and those of the lowly virulent strains. Thus, these latter strains have been designated as *Burkholderia pseudomallei*-like organisms since they demonstrate significant differences in exoenzyme production, hamster virulence and 16S rRNA gene sequences.

INTRODUCTION

Burkholderia pseudomallei, the causative agent of melioidosis [1–3], is a bacterial pathogen endemic to Southeast Asia, Northern Australia and temperate regions that border the equator [1, 4]. While melioidosis presents a significant health threat to people indigenous to these geographical domains, the clinical manifestations of this disease are rarely observed in the Western Hemisphere [4, 5]. The organism may be routinely isolated from environmental niches such as stagnant waters, moist soils and rice paddies [6–8]. Acquisition of melioidosis is thought to occur via the inoculation of damaged surface tissues with the contaminated soils or water, or by the inhalation and aspiration of infectious dust particles [4, 8, 9].

Melioidosis manifests itself as acute, sub-acute and chronic illnesses [4]. The acute disease is rapidly fatal without treatment, and even chemotherapeutic intervention in these cases offers a poor prognosis for the patient [4, 10, 11]. Sub-acute melioidosis is best described as a prolonged febrile illness characterized by multi-organ involvement, systemic abscess formation and bacteraemia. This too is fatal if left untreated [11].

Chronic melioidosis is thought to be the most common presentation of this disease [10]. Diagnosis is most often achieved by post-mortem examination of infected tissues, or when clinical indications present themselves upon activation of the disease by a traumatic event. Accumulating evidence suggests that physiological abnormalities such as diabetes mellitus and renal disease may predispose a host to *Burkholderia pseudomallei* infections [1, 12, 13].

Our laboratory has initiated studies to define the pathogenesis of disease due to *Burkholderia pseudomallei*. Although several putative virulence

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determinants have been implicated in the pathogenesis of melioidosis, it is only recently that we have begun to elucidate the roles of such factors in the establishment and maintenance of these infections. One of our approaches has been to characterize the role in disease for those exoproducts secreted by *Burkholderia pseudomallei* strains which include a thermolabile toxin, protease, lipase, lecithinase and a water-soluble siderophore [8, 14–19]. Cell-associated factors such as lipopolysaccharide [LPS], flagella, capsule and pili are also considered to be of potential significance in the pathogenesis of this organism [8, 15, 20, 21]. Studies from our laboratory have identified LPS and flagella as protective antigens, and mounting evidence suggests that these may be useful in the development of a vaccine for protection against *Burkholderia pseudomallei* infections [20, 22, 23].

A second approach which we have taken to define the pathogenesis of *Burkholderia pseudomallei* infections has been to compare the phenotypes of *Burkholderia pseudomallei* strains which are virulent for animals with the phenotypes of *Burkholderia pseudomallei* strains which are avirulent for animals. Previous reports in the literature suggest that *Burkholderia pseudomallei* strains exist which are avirulent for animals [24–26]. Immunization of mice with these strains decreased their susceptibility to virulent strains of *Burkholderia pseudomallei* but did not alter the progression of established disease [27]. Further, McCormick and colleagues [25] reported on a wound infection in a man injured in a farming accident in Oklahoma by an indigenous *Burkholderia pseudomallei*-like organism isolated from the soil which was avirulent in guinea-pigs. The present studies were undertaken to examine the phenotypic and genotypic differences between *Burkholderia pseudomallei* strains which are virulent in animals versus those which are avirulent in animals. The results obtained indicate that these avirulent *Burkholderia pseudomallei*-like strains may represent a separate species of *Burkholderia*.

METHODS

Bacterial strains and cell lines

Burkholderia pseudomallei strains were obtained from Dr D. A. B. Dance and Dr Mike Smith, Wellcome–Mahidol–Oxford Tropical Medicine Research Programme, Bangkok, Thailand. All stock cultures were maintained in 10% skim milk or 20% glycerol suspensions and stored at −70 °C. Cell lines were obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in cell culture media containing a standard antibiotic mixture (100 U penicillin, 100 μg streptomycin and 250 μg of amphotericin B per ml; Sigma Chemical Co., St Louis, MO) plus 10% fetal bovine calf serum (FBS; Gibco Canada Inc., Mississauga, Ontario, Canada) at 37 °C in 5% CO_2. Culture media for HeLa cells was Dulbecco's modified Eagle's medium (DMEM; Gibco Canada Inc.).

Specialized media

Modified Ashdown's selective media (Ashdown's selective media plus 100 μg/ml streptomycin and 15 μg/ml gentamicin; MASM) was used to maintain and differentiate *Burkholderia pseudomallei* strains in routine laboratory use [28]. TSBDC culture medium was prepared as follows: 60 g of tryptic soy broth (TSB; Difco Laboratories Inc., Detroit, MI) was added to 180 ml of distilled water and stirred for 4–6 h at room temperature with 10 g of Chelex 100 resin (Bio Rad Laboratories Inc., Richmond, CA). The solution was then dialysed overnight at 4 °C against 2·0 l of distilled water. The dialysate was autoclaved, and filter sterilized solutions of glycerol and monosodium glutamate (MSG; Sigma Chemical Co.) was added to final concentrations of 1% glycerol and 50 mM MSG. CASDC culture medium was prepared as per TSBDC except that casein hydrolysate (Difco Laboratories Inc.), and not TSB, was used as the nutrient base.

Isolation of LPS and supernatant antigens

Homogeneous preparations of LPS derived from *Burkholderia pseudomallei* strains E264 and 316c were obtained as previously described by Brett and colleagues [22]. The E264 and 316c supernatant antigens for use in hamster lethality assays and antibody production were prepared as follows. Briefly, 250 ml of TSBDC culture medium in 2·0 l flasks was inoculated with either *Burkholderia pseudomallei* E264 or 316c. The cultures were incubated at 37 °C and 250 rpm for 24 h, following which the cells were removed by centrifugation at 8000 g for 20 min. The culture supernatants were then filter sterilized, and lyophilized as a concentration step. The solids were resolubilized in phosphate buffered saline (PBS) to a

total protein concentration of 200 mg/ml, and stored at −20 °C until required for use.

Detection of secreted products

Substrate specific assay media were inoculated with *Burkholderia pseudomallei* strains grown on MASM agar. Briefly, protease activity was demonstrated by a zone of clearing around individual colonies on 3% skim milk agar plates, while siderophore production was observed as an orange halo that surrounded the bacterial colonies on a chrome azurol S (CAS) based plate assay [29]. Lipase and lecithinase activities were assessed essentially as described [30] using a medium of the following composition: tryptic soy agar 480 g, yeast extract 180 g, dextrose 24 g, $CaCl_2.H_2O$ 8·4 g, $MgCl_2.6H_2O$ 0·84 g, 0·7% $ZnSO_4.H_2O$ 12 ml, distilled H_2O 12 l. The components were dissolved by boiling and the pH adjusted to 7·5. Lecithinase production was seen as a cloudy (opaque) zone around the positive producing strains, while lipase activity was demonstrated by an iridescent sheen which surrounded colonies on the egg yolk agar (EYA). All plate assays were examined for substrate specific activity following incubation for 48 h at 37 °C.

Cytotoxicity assays

The cytotoxic activity of supernatant antigens from *Burkholderia pseudomallei* strains was assessed using a HeLa tissue culture assay. Two ml aliquots of CASDC culture medium in 10 ml snap cap tubes were inoculated with individual environmental and clinical strains and incubated at 37 °C and 250 rpm for 24 h. The cells were removed by centrifugation, and the culture supernatants filter sterilized for immediate use. HeLa cells were seeded in 96 well plates at 5×10^4 cells/well in DMEM plus 10% FBS and grown to confluency at 37 °C and 5% CO_2. The culture medium was removed from the wells, and CASDC supernatants (serially diluted in DMEM prior to addition to the tissue culture) were added in duplicate: 8×, 16×, 32×, 64×, 128× and 256×. The plates were then incubated for 48 h at 37 °C at 5% CO_2. Cell viability was subsequently determined by the MTT-based colorimetric assay described in Bio Rad Bulletin 1203 (Bio-Rad Laboratories). The % cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = [1 - (\text{O.D. toxin treated})/(\text{O.D. untreated control})] \times 100\%.$$

Antibody production

Antibodies to *Burkholderia pseudomallei* E264 and 316c supernatant antigens were prepared by administration of Freund's complete adjuvant (FCA; BRL Life Technologies Inc., Gaithersburg, MD) emulsified with filter sterilized preparations of the concentrated culture supernatants. Rabbits (New Zealand White, 2–2·5 kg) each received an intramuscular injection of 0·50 ml of the emulsion in both their right and left hind thigh muscles. Each injection contained 1 mg of total protein per ml. The rabbits received a booster injection on days 14 and 28 post primary immunization with Freund's incomplete adjuvant (FIA; BRL Life Technologies Inc.) as the emulsifying agent. Animals were exsanguinated by cardiac puncture under anaesthesia at day 42, and serum samples were collected and stored at −70 °C until required for use.

Western immunoblotting

Supernatant antigens were subjected to SDS–PAGE using a 4% stacking and 12·5% separating slab gel system [31]. Electrophoretic transfer of antigens to a nitrocellulose membrane (Bio-Rad Laboratories) was performed as described by Towbin [32] using a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories) operating at 40 V overnight at 4 °C. Immunoblotting was performed as described by Catty [33] and developed with an HRP Color Development Reagent (Bio-Rad Laboratories).

Assessment of *Burkholderia pseudomallei* strain and antigen lethality in Syrian golden hamsters

The lethality of *Burkholderia pseudomallei* strains was assessed in a Syrian golden hamster animal model of infection (6–8 weeks, Charles River, Canada) using late log phase bacteria grown in TSBDC culture media at 37 °C. Briefly, hamsters were inoculated intraperitoneally (IP) with 100 μl of one of a number of serial dilutions of the log phase cultures adjusted appropriately with sterile PBS (five hamsters per dilution). Strains E300, 316c and 1026b were administered at doses consisting of approximately 1×10^0, 1×10^1, 5×10^1, 1×10^2 and 1×10^3 CFU. A control group received PBS. Similarly, groups of hamsters were inoculated with approximately 1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 CFU of strains E264 and E275. The animals were then monitored for

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4 weeks for signs of morbidity and mortality. The LD_{50} values at a number of time points were calculated as previously described by Reed and Muench [34].

Purified LPS from *Burkholderia pseudomallei* strains E264 and 316c was administered to hamsters in order to assess the lethal effects of these molecules. Briefly, 5 groups of hamsters containing 5 animals per group were challenged IP with 100 μl aliquots of 1 of 4 doses of filter sterilized 316c LPS diluted in PBS: 0·1 mg/ml, 1·0 mg/ml, 5·0 mg/ml, 10 mg/ml. The remaining group received PBS as a control. The same procedure was repeated for E264 samples. The animals were monitored for a period of 7 days for signs of morbidity and mortality. The same procedure was utilized to test the lethal effects of the culture supernatant antigens isolated from the same two *Burkholderia pseudomallei* strains. Four groups of hamsters were administered 100 μl of three different concentrations of 316c filter sterilized total supernatant protein solubilized in PBS intraperitoneally: 0·2 mg/ml, 20 mg/ml and 200 mg/ml. Again, the remaining group received PBS as a control, and the procedure was repeated for E264 samples. The animals were similarly monitored for 7 days post challenge, and LD_{50} values were calculated as previously described [34].

Amplification and sequence analysis of 16S rDNA

Genomic DNA was isolated as previously described [35] and used as a template to PCR-amplify a 320 bp region of the 16S rRNA gene (16S rDNA) from *Burkholderia pseudomallei*. The oligodeoxyribonucleotide primers used in the PCR were designed based on the previously published *Burkholderia pseudomallei* 16S rDNA sequence [3]. The primer 16S 325–345 (5′ AGACACGGCCCAGACTCCTAC 3′) corresponds to nucleotide 325–345 and the primer 16S 635–645 (5′ CAGTCACCAATGCAGTTCCCA 3′) is the reverse complement of nucleotide 625–645 [3]. The 16S rDNA was PCR-amplified in a 100 μl reaction mix containing 10 ng of genomic DNA, 1 × PCR Buffer II (Perkin Elmer Cetus, Norwalk, CT), 2 mM $MgCl_2$, 40 μM of each dNTP, 0·2 μm of each primer and 2·5 U of AmpliTaq DNA Polymerase (Perkin Elmer Cetus). The PCR mixture was transferred to a GeneAmp PCR System 9600 (Perkin Elmer Cetus) thermal cycler for 25 cycles of a 3-temperature cycling protocol (95 °C for 15 s, 62 °C for 30 s, 72 °C for 30 s). The size and purity of the PCR product was assessed by electrophoresis of 20 μl of the reaction mixture through a 1·2% agarose gel followed by visualization of ethidium bromide-stained fragments with a UV light source. The PCR product was isolated from the reaction mixture with the Magic PCR Preps DNA Purification System (Promega, Madison, WI). The AmpliCycle Sequencing Kit (Perkin Elmer Cetus) was used to sequence the PCR product directly with 16S 325–345 and 16S 625–645. The sequence reactions were placed in the GeneAmp PCR System 9600 for an initial step of 95 °C for 1 min, followed by 25 cycles of a 3-temperature cycling protocol (95 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min). The sequencing products were separated on a 6% acrylamide gel and subsequently visualized by autoradiography.

RESULTS

Characterization of secretion profiles

The secretion profiles of 20 environmental and clinically derived isolates of *Burkholderia pseudomallei* were examined using substrate specific bioassays and HeLa tissue culture techniques. The results of these studies under defined conditions demonstrated that there were two phenotypically distinct secretion profiles which were characteristic of the 20 strains tested. For 10 of the 20 isolates examined, the presence of proteolytic (PRT) and siderophore (SID) activities along with low to moderate *in vitro* cytotoxic (CTX) activities were demonstrated for these organisms (Table 1). In contrast, 9 of the 20 strains demonstrated PRT, lipase (LIP), lecithinase (LET) and SID activities as well as high CTX activity in the HeLa tissue culture assays. Only *Burkholderia pseudomallei* strain 199a was found to deviate from either one of these phenotypic patterns. This isolate was characterized by PRT, SID and LIP activities as well as exhibiting moderate CTX properties (Table 1).

Colony morphologies on MASM

It was found that on MASM agar, strains that were representative of the two distinct secretion phenotypes could be differentiated from one another based on their colony morphology. In fact, there was an excellent correlation between a given colony morphology and a particular secretion profile. Colonies derived from strains that were positive for PRT/

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Table 1. *Characterization of the secretion profiles of 20 environmental and clinical* Burkholderia pseudomallei *strains*

Strain	Protease*	Lipase†	Lecithinase†	Siderophore‡	HeLa cytotoxicity (% killing)§	Source
E202	+	+	+	+	85	Environmental
E210	+	−	−	+	0	Environmental
E250	+	−	−	+	21	Environmental
E251	+	+	+	+	87	Environmental
E252	+	+	+	+	77	Environmental
E264	+	+	+	+	84	Environmental
E265	+	+	+	+	87	Environmental
E266	+	+	+	+	77	Environmental
E275	+	+	+	+	75	Environmental
E276	+	+	+	+	89	Environmental
E277	+	−	−	+	0	Environmental
E284	+	−	−	+	0	Environmental
E285	+	+	+	+	100	Environmental
E300	+	−	−	+	0	Environmental
E304	+	−	−	+	0	Environmental
199a	+	+	−	+	50	Clinical
307e	+	−	−	+	28	Clinical
316c	+	−	−	+	28	Clinical
319a	+	−	−	+	43	Clinical
1026b	+	−	−	+	43	Clinical

* Protease activity determined on 3% skim milk agar plates.
† Lipase and lecithinase activity determined on EYA plates [17].
‡ Siderophore activity determined on CAS agar plates [32].
§ Values are the result of CASDC culture supernatants (16 × dilution) incubated with HeLa tissue cultures. % killing = [1 − (0.D. toxin treated)/(O.D. untreated control)].

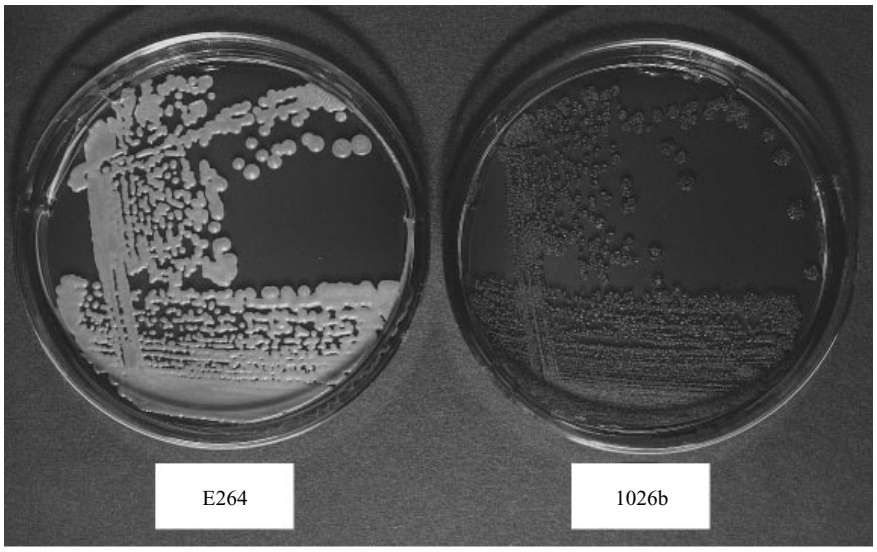

Fig. 1. *Burkholderia pseudomallei* colony morphologies as demonstrated on MASM agar. PRT/LIP/LET/SID phenotype: E264. PRT/SID phenotype: 1026b. Plates were incubated for 48 h at 37 °C and were subsequently placed at room temperature for 5 days.

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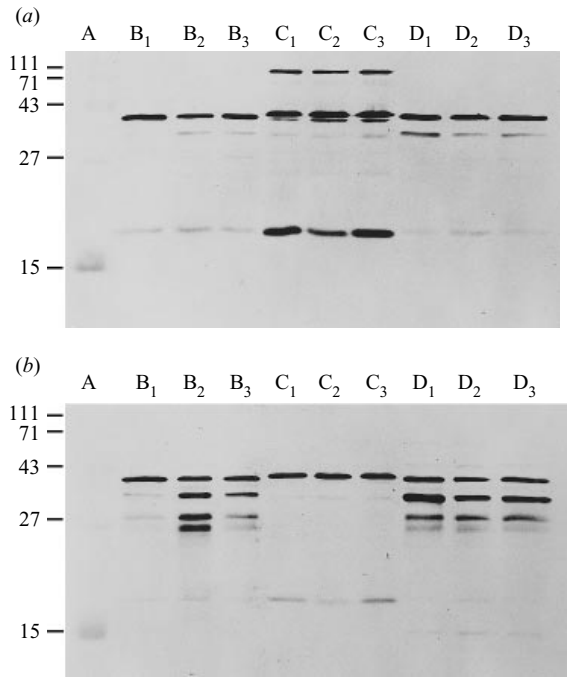

Fig. 2. (*a*) Immunoblot profiles of *Burkholderia pseudomallei* secretion products. Lanes containing supernatant antigens were loaded with 30 μl of unconcentrated TSBDC samples and reacted with a 1/1500 dilution of E264 specific polyclonal antiserum. (A) Apparent M_r of pre-stained protein markers (BRL Laboratories Inc.) were phosphorylase b [111 kDa], bovine serum albumin (71 kDa), ovalbumin (43 kDa), carbonic anhydrase (27 kDa), lysozyme (15 kDa). (B_1–B_3) Clinical strains: 199a, 316c, 1026b. (C_1–C_3) Environmental PRT/LIP/LET/SID strains: E264, E266, E275. (D_1–D_3) Environmental PRT/SID strains: E250, E277, E300. No reactivity was observed with pre-immune serum in all cases (data not shown). (*b*) Immunoblot profiles of *Burkholderia pseudomallei* secretion products. Lanes containing supernatant antigens were loaded with 30 μl of unconcentrated TSBDC samples and reacted with a 1/1000 dilution of 316c specific polyclonal antiserum. Lanes A–D are as described in (*a*). No reactivity was observed with pre-immune serum in all cases (data not shown).

LIP/LET/SID activities had a light pink coloration and a smooth appearance, whereas the strains that demonstrated PRT/SID activities presented a characteristic dry, deeply wrinkled colony morphology with a dark red pigmentation (Fig. 1). *Burkholderia pseudomallei* strain 199a was shown to exhibit the latter morphology when incubated on MASM agar.

Immunoblot analysis of supernatant antigens

Immunoblotting techniques were used to estimate the degree of cross-reactivity and the relative mobilities (M_r) of the supernatant antigens from a variety of strains. Three samples from each of the clinical [199a, 316c, 1026b], environmental PRT/SID [E250, E277, E300] and environmental PRT/LIP/LET/SID [E264, E266, E275] strains were examined by this method. As predicted, the PRT/LIP/LET/SID strains, when reacted with E264 polyclonal antiserum (prototypic PRT/LIP/LET/SID strain) demonstrated a unique antigen profile as compared to the clinical and environmental PRT/SID isolates. Characteristic of these strains were three antigens demonstrating M_r of approximately 87, 40 and 37 kd respectively (Fig. 2*a*).

When 316c polyclonal antiserum [prototypic PRT/SID strain] was used to examine the same nine supernatant products, similar results were achieved. The clinical and environmental PRT/SID strains demonstrated a multiple banding pattern ranging from an M_r of 23–38 kd; whereas the PRT/LIP/LET/SID strain specific supernatants either railed to react or did so weakly at these positions (Fig. 2*b*). Results of these immunoblots confirm that a number of cross-reactive epitopes are shared between the two phenotypically distinct groups; however, their antigen profiles are such that the PRT/SID and PRT/LIP/LET/SID strains can be differentiated from one another by this technique. In all cases Coomassie blue staining of SDS–PAGE supernatant antigen profiles yielded similar results to those obtained by immunoblot analysis (data not shown). Neither the E264-P1 passage strain or strains isolated post mortem demonstrated altered secretion phenotypes than those expected by immunoblot analysis (data not shown).

Strain-specific lethality in Syrian golden hamsters

In previous studies, we found that the virulence of *Burkholderia pseudomallei* strains 316c (PRT/SID phenotype: $< 10^2$ CFU) and E264 (PRT/LIP/LET/SID phenotype: $> 10^6$ CFU) was markedly different for Syrian golden hamsters when administered by the IP route. Based on these findings, we predicted that an inoculum of 10^3 CFU of *Burkholderia pseudomallei* would be 100% lethal in 48 h for hamsters receiving PRT/SID phenotypes while non-lethal for those receiving PRT/LIP/LET/SID phenotypes. Using 5 hamsters in a group, 20 different groups of animals were inoculated IP with 1 of the 20 different *Burkholderia pseudomallei* strains. After 48 h, 10/10 of the strains that demonstrated a PRT/SID phenotype were 100% lethal for the hamsters (as was strain 199a: PRT/LIP/SID), while the animals inoculated

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Table 2. *Correlation between* Burkholderia pseudomallei *phenotypic profiles and mortality at 48 h in golden hamster lethality assays*

Strain	Secretion phenotype	Hamster mortality at 48 h* (inoculum = 10^3 CFU; $n = 5$)
E202	PRT/LIP/LET/SID†	0
E210	PRT/SID‡	5
E250	PRT/SID	5
E251	PRT/LIP/LET/SID	0
E252	PRT/LIP/LET/SID	0
E264	PRT/LIP/LET/SID	0
E265	PRT/LIP/LET/SID	0
E266	PRT/LIP/LET/SID	0
E275	PRT/LIP/LET/SID	0
E276	PRT/LIP/LET/SID	0
E277	PRT/SID	5
E284	PRT/SID	5
E285	PRT/LIP/LET/SID	0
E300	PRT/SID	5
E304	PRT/SID	5
199a	PRT/LIP/SID§	5
307e	PRT/SID	5
316c	PRT/SID	5
319a	PRT/SID	5
1026b	PRT/SID	5

* Groups of hamsters with 5 in each were inoculated IP with 1 of 20 different strains of *B. pseudomallei* and observed for signs of morbidity and mortality at 48 h post inoculation.
† PRT/LIP/LET/SID: protease/lipase/lecithinase/siderophore activities associated with these strains.
‡ PRT/SID: protease/siderophore activities associated with these strains.
§ PRT/LIP/SID: protease/lipase/siderophore activities associated with this strain.

with PRT/LIP/LET/SID strains displayed no signs of morbidity or mortality (Table 2). Thus, a correlation can be drawn between *Burkholderia pseudomallei* strain-specific secretion profiles and strain-specific virulence for hamsters.

Determination of minimum LD_{50} values for highly virulent and lowly virulent strains in Syrian golden hamsters

The LD_{50} values for representative PRT/SID and PRT/LIP/LET/SID strains were calculated in order to assess the relative virulence of each of the two phenotypes in hamsters. At 48 h post inoculation of the hamsters with PRT/SID strains, the LD_{50} value for E300 was determined to be < 2 CFU, while the LD_{50} values for 316c and 1026b were < 10 and < 12 CFU, respectively (Table 3). Blood drawn from the hamsters at time of death was cultured for the presence of *Burkholderia pseudomallei*, and the results demonstrated that each of the strains was able to elicit an overwhelming bacteraemia in animals receiving an inoculum as low as 10^1 CFU. Plate counts displayed numbers in excess of 10^4 CFU/ml of blood in all three cases (Table 3).

The LD_{50} values calculated for the PRT/LIP/LET/SID strains in the same time frame were markedly different, however, with the LD_{50} of E264 and E275 determined to be $1{\cdot}8 \times 10^6$ and $1{\cdot}3 \times 10^7$ CFU respectively (Table 3). Blood samples from the 10^3 CFU inoculum groups (which showed no signs of morbidity or mortality at this point) were devoid of *Burkholderia pseudomallei*; whereas samples analysed from the hamsters which had succumbed to infection ($> 10^6$ CFU) displayed counts similar to the PRT/SID phenotypic strains. In a separate study, a single passage strain (E264: isolated from the liver of a hamster succumbing to infection at 19 days post inoculation) was similarly administered to hamsters. The results demonstrated that the PRT/LIP/LET/SID passage strain, although more virulent at 7 days as compared to its parental strain, was not as virulent as the PRT/SID strains at 48 h (Table 3). This study was terminated at 7 days since the goal was to assess only the ability of this strain to mimic the acute infections elicited by the highly virulent strains.

It was also shown in these studies that dose dependent mortality could be achieved with the PRT/LIP/LET/SID strains. We were able to mimic sub-acute group infections by administering the appropriate inoculum of bacteria (Table 3).

Assessment of supernatant antigen and LPS lethality in hamsters

In order to assess the lethal effects of biological components associated with PRT/SID and PRT/LIP/LET/SID phenotypes, purified LPS and concentrated culture supernatants were administered IP to hamsters. The results demonstrated that the LD_{50} for purified LPS from both 316c and E264 was 1000 mg per hamster, while the LD_{50} for concentrated culture supernatants was > 200 mg/ml total protein. This suggests that the $> 10^5$ fold difference in virulence demonstrated between PRT/SID and PRT/LIP/LET/SID strains is probably not a result of unique

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Table 3. *Estimation of minimum LD_{50} values for representative PRT/SID and PRT/LIP/LET/SID specific phenotypes*

Strain	LD_{50} (CFU)*				Blood cultures† (CFU/ml)
	48 h	7 days	14 days	28 days	
E264	$1{\cdot}8 \times 10^6$	$3{\cdot}4 \times 10^5$	$8{\cdot}5 \times 10^4$	$2{\cdot}6 \times 10^4$	0‡
E264-P1‖	$> 1{\cdot}2 \times 10^6$	$< 1{\cdot}2 \times 10^3$	—	—	0‡
E275	$1{\cdot}3 \times 10^7$	$4{\cdot}1 \times 10^6$	$9{\cdot}4 \times 10^4$	$3{\cdot}4 \times 10^4$	0‡
E300	< 2	—	—	—	$1{\cdot}0 \times 10^5$§
316c	< 10	—	—	—	$1{\cdot}7 \times 10^4$§
1026b	< 12	—	—	—	$5{\cdot}3 \times 10^6$§

* LD_{50} values were calculated at various time points as described by Reed and Muench [30].
† Blood cultures were plated to MASM agar to determine the bacterial loads of the hamsters.
‡ Values represent counts from the 10^3 inoculum groups bled at 48 h.
§ Values represent counts from the 10^1 inoculum groups bled at time of death (< 48 h).
‖ E264 passage strain isolated from liver tissue at 19 days.
—, 100% mortality.

toxic properties associated with one group or the other.

Sequencing of 16S rRNA genes

Yabuchi and colleagues [3] previously determined the sequence of the 16S rDNA from ATCC 23343, a clinical isolate of *Burkholderia pseudomallei*. We determined the sequence of a 320 bp portion of the 16S rDNA (nucleotide 325–645) from highly virulent and lowly hamster-avirulent strains of *Burkholderia pseudomallei* and compared them to the published ATCC 23343 16S rDNA sequence. The sequence obtained from two clinical PRT/SID strains (316c and 1026b) and two environmental PRT/SID strains (E300 and E304) were identical to each other and to the ATCC 23343 sequence (Fig. 3*a*). In contrast, the sequence of the two environmental PRT/LIP/LET/SID strains (E264 and E275) contained three nucleotide differences relative to ATCC 23343 and the PRT/SID strains (Fig. 3*a*). These nucleotide differences were identical in both E264 and E275 and corresponded to nucleotide positions 458, 469 and 474 in the published ATCC 23343 16S rDNA sequence [3]. Thus, the partial 16S rDNA sequence from highly virulent strains was identical to the previously published *Burkholderia pseudomallei* sequence while there were three nucleotide differences in the 16S rDNA from lowly virulent strains. In addition, the 16S rDNA sequence obtained from E264 and E275 did not have an exact match in the GenBank database (release 91.0).

The region encompassing nucleotides 325–645 from the 16S rRNA genes was chosen for sequencing based upon the reported hypervariable nature of this domain throughout the *Burkholderia* spp. [36]. Figure 3*b* thus serves to exemplify further the presence of nucleotide sequence dissimilarities in this region by comparing the same 320-bp portions from a variety of *Burkholderia* spp. with the ATCC 23343 sequence [3, 36–38].

Biochemical profiles of *Burkholderia pseudomallei* strains

The biochemical profiles of all the *Burkholderia pseudomallei* strains employed in the present studies were obtained using API NFT strips. The profiles of the virulent *Burkholderia pseudomallei* strains were consistent with the published documentation on *Burkholderia pseudomallei* isolates [39]; however, the profiles of the avirulent *Burkholderia pseudomallei* strains differed in one parameter, i.e. these strains assimilated L-arabinose. While this characteristic on its own does not exclude these strains from *Burkholderia pseudomallei* species status, it is a consistent observation and one which could be used in clinical laboratories to differentiate *Burkholderia pseudomallei*-like organisms from true *Burkholderia pseudomallei*.

DISCUSSION

Previous reports in the literature suggest that *Burkholderia pseudomallei* strains exist which are avirulent for animals [24–26, 40]. The present studies were undertaken to examine the phenotypic and

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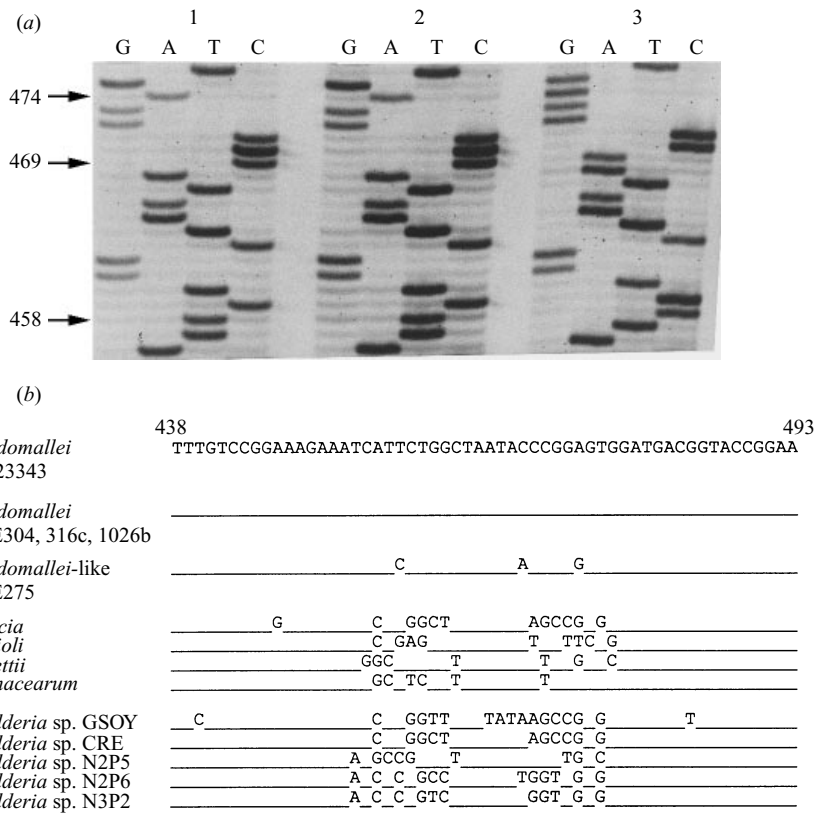

Fig. 3. (*a*) DNA sequence ladders of the 16S rDNA from hamster-virulent and hamster-avirulent *Burkholderia pseudomallei* strains. Lanes: 1, E300; 2, 316C; 3, E275. The arrows point to the nucleotide differences between the hamster-virulent strains (E300 and 316C) and the hamster-avirulent strain (E275). The numbers to the left of the arrows correspond to the published *Burkholderia pseudomallei* 16S rDNA sequence [3]. (*b*) Sequence alignment analysis of a hypervariable region of the 16S rRNA genes from various *Burkholderia* spp. compared to that of *B. pseudomallei* ATCC 23343 [3, 36–38].

genotypic differences between *Burkholderia pseudomallei* strains which are highly virulent in animals versus those which are lowly virulent in animals with the anticipation that this approach would allow us to further define the pathogenesis of *Burkholderia pseudomallei* infections. We have examined 20 clinical and environmentally derived isolates of *Burkholderia pseudomallei*. By utilizing a variety of biochemical and tissue culture techniques, together with an animal model of infection, we have been successful in defining two phenotypically distinct profiles that are associated with differential virulence for Syrian golden hamsters.

Characteristic of the highly virulent phenotype was the ability of these organisms to secrete substances responsible for PRT and SID activities while simultaneously producing relatively low CTX activity. In contrast, the lowly virulent phenotype demonstrated PRT, LIP, LET and SID activities in conjunction with significantly higher CTX activity as compared to the highly virulent strains. The only strain to deviate from these profiles was *Burkholderia pseudomallei* 199a. Further investigation of this strain, however, will be required in order to determine the clinical significance associated with the LIP activity of this isolate. The MASM agar was also useful for demonstrating morphological differences between the highly virulent and lowly virulent strains. This is the first report of a correlation between colony morphology and the virulence associated with *Burkholderia pseudomallei* strains.

To date, a total of 35 clinical isolates have been examined for the expression of biologically active exoproducts, and all but 199a demonstrate phenotypic profiles characteristic of virulent strains. These findings contradict the results of a similar survey which suggested that 96–97% of clinical strains produce LIP, LET and PRT activities [41]. The discrepancies between our study and this previous study can probably be explained by differences in media composition or perhaps incubation temperature. We have found, for example, that strains negative for lipase and phospholipase C activity using our egg yolk media and an incubation temperature of

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37 °C can be induced to produce lipase when incubated at 25 °C (unpublished observations). It was also demonstrated from the phenotypic profiles of the environmental isolates that acquisition of acute cases of melioidosis from environmental reservoirs is a reasonable hypothesis since both highly virulent and lowly virulent strains are present in the environment [2, 27, 39].

Results from immunoblot analyses of the culture supernatants confirmed the presence of two distinct phenotypic profiles. Regardless of whether the supernatants were reacted with antiserum raised against highly virulent or lowly virulent strains, there were always two easily distinguishable antigen profiles which could be attributed to the differential secretion products synthesized by these organisms. Since a significant amount of cross-reactivity was demonstrated between the different strains, one might have expected to observe a higher degree of homology in the relative mobility of the secretion products. This anomaly suggests that while the phenotypically distinct *Burkholderia pseudomallei* strains do share similar biological activities, the cellular products responsible for such activities may not be physically identical. Preliminary studies in our laboratory indicate that this may be the case since the proteases of strains 316c and E264 appear to be structurally dissimilar (unpublished observations).

As Syrian golden hamsters are exquisitely sensitive to highly virulent strains of *Burkholderia pseudomallei* [9, 24, 26, 40, 42], these animals were chosen to confirm the virulence phenotypes of the 20 strains characterized in this communication. When the hamsters were inoculated with the 11 strains predicted to be highly virulent, 5/5 of the hamsters in each of the study groups succumbed to infection within 48 h, whereas the 9 strains predicted to have a lowly virulent phenotype had no effect on the hamsters in the same time frame. These results show that a correlation between secretion profiles and virulence exists (Tables 1, 2).

The LD_{50} values for representative highly virulent and lowly virulent strains of *Burkholderia pseudomallei* were estimated in separate hamster lethality assays. The results demonstrate that a $> 10^5$ fold difference (at 48 h) in the relative virulence properties of the two phenotypic groups is characteristic of these organisms. Previous studies by Dannenberg and colleagues demonstrated that the route of inoculation had no bearing on the outcomes of such studies when similar strains were employed [24, 40]. Hamsters inoculated with highly virulent strains were unable to survive past 48 h even when administered doses as low as $< 10^1$ CFU. Bacterial counts from the blood of these animals at time of death displayed a marked bacteraemia in all cases ($> 10^4$ CFU/ml). Mortality in these situations was distinguished by the presence of nasal, oral and ocular secretions containing blood. The lethal effects of acute melioidosis caused by virulent strains can thus best be described as consistent with the clinical manifestations of a septicotoxaemic group illness [40]. In many respects, the pathogenesis in these cases resembles that associated with plague and anthrax [43, 44].

Inoculum dependent mortality was observed when lowly virulent strains were administered intraperitoneally to the hamsters. In studies where the hamsters were challenged with $> 10^6$ CFU, the animals succumbed to infection in a similar fashion to that described for highly virulent strains. No lesions were demonstrated on the major organs, and $> 10^4$ CFU/ml were isolated from the blood at time of death. If, however, the lowly virulent organisms were administered at a lower inoculum, one could establish a sub-acute group of illness in these animals. Hamsters that died from these infections displayed clinical manifestations commonly associated with sub-acute melioidosis. Post-mortem examinations of the liver, kidneys, lungs, heart and spleen displayed multiple abscesses at these sites; however, no visible pathology was observed in the brains of these animals. All these tissues, however, were positive for viable organisms when cultured appropriately. In many instances, 2–3 days prior to death, hamsters with these sub-acute group illnesses developed hind leg paralysis and uncontrollable shaking, sequelae which have been previously reported by Nigg and colleagues [42] following administration of culture filtrates from selected *Burkholderia pseudomallei* strains.

Such studies raise the question of whether or not the secretion products from these organisms are a critical factor in the pathogenesis of acute melioidosis. For example, based upon their biologically active secretion repertoire, lowly virulent organisms should be qualified at establishing acute illnesses [at much lower inoculums than those demonstrated] if this was not the case. Also, the concentrated culture supernatants from both highly virulent and lowly virulent strains were shown to be ineffective at eliciting any of the clinical manifestations associated with an acute group illness. However, in preliminary studies we have found that transposon mutants incapable of trans-

locating exoproducts across their outer membranes exhibit somewhat attenuated virulence as compared to their highly virulent parental strains (unpublished results). Information obtained from ongoing studies utilizing the transposon mutants should help to clarify the clinical significance of these exoproducts secreted by *Burkholderia pseudomallei* strains.

The DNA sequences of the 16S rRNA genes of the highly virulent *Burkholderia pseudomallei* strains were identical to the published sequences for *Burkholderia pseudomallei* [3] while differences were revealed between the published sequences and those of the lowly virulent strains. Comparison of the 320-bp 16S rDNA nucleotide sequences from selected strains employed in this study with similar regions derived from various other *Burkholderia* spp. further demonstrated the identity of the highly virulent strains as *B. pseudomallei* isolates. The same correlation could not be made for the lowly virulent strains and thus raises the question as to the appropriate species designation for these organisms. Further sequence analysis of the genome should, however, help to shed some light on this matter. Thus, we have designated these latter strains as *Burkholderia pseudomallei*-like organisms in that though phenotypically similar to true *Burkholderia pseudomallei* strains, they demonstrate significant differences in exoenzyme production, hamster virulence and 16S rRNA gene sequences. Based upon this information, the continued use of these organisms in comparative pathogenesis studies is problematic. It could very well be that these organisms might be appropriately reclassified as a species separate from *Burkholderia pseudomallei*. Further, the ability to assimilate arabinose could be used by the clinical microbiology laboratory as a simple test to classify these strains.

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