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Intracellular Existence of *Burkholderia pseudomallei*

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ABSTRACT

The chronic and latent forms of melioidosis, as well as its recrudescence, are facets of the disease that are clearly influenced by the ability of *Burkholderia pseudomallei* to invade eukaryotic cells and survive in the intracellular milieu. We have initiated investigations into the intracellular existence of this pathogen.

Burkholderia pseudomallei is a pathogen with the ability to invade both professional and non professional phagocytes. We have demonstrated that *B. pseudomallei* is able to invade and replicate in A549 type II pneumocytes. Additionally, we have established that a phagosome-lysosome fusion occurs once the pathogen has been internalized in membrane bound vacuoles.

One bactericidal challenge that *B. pseudomallei* encounters after the phagosome-lysosome fusion, is an acidic environment. We have demonstrated that *B. pseudomallei* secretes an extracellular polysaccharide in response to acidic challenge. We have isolated this EPS and studies are currently underway to determine its monosaccharide components.

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To my mother,

For having an eternal grace and dignity that is rarely seen.

For reminding me that laughter is always the best medicine.

For her strength and beauty and the encouragement she showed me.

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Ap - ampicillin

C – Celsius

CF – cystic fibrosis

cfu – colony forming units

Cm – chloramphenicol

CTX – cytotoxic activity

Da – daltons

DMSO – dimethylsulfoxide

DNA – deoxyribonucleic acid

ELISA – enzyme linked immunosorbant assay

EM – electron microscopy

EP – ethanol precipitate

EPS – exopolysaccharide

FBS – fetal bovine serum

hr – hour

IFNγ – gamma interferon

g – gram

GFP – green fluorescent protein

IgG – immunoglobulin G

KDO – 3-deoxy-D-mann-octulosonic acid

Km - kanamycin

kg – kilogram

l – litre

LB – luria bertani

LET – lecithinase

LIP – lipase

LPS – lipopolysaccharide

LOS – lipooligosaccharide

M – moles per litre

mAb – monoclonal antibody

min. – minutes

mg – milligrams

MIC – minimum inhibitory concentration

ml – millilitre

mM – millimolar

nM – nanomolar

MOI – multiplicity of infection

Mr – molecular weight

Noc. – nocodazole

NHS – normal human sera

nm – nanometers

OD – optical density

O-PS – O-antigenic polysaccharide

PBS-T – PBS-tween

PMN – polymorphonuclear neutrophils

PRT – protease

RAPD – random amplified polymorphic DNA analysis

RFLP – restriction length fragment polymorphisms

RNA – ribonucleic acid

rRNA – ribosomal ribonucleic acid

RPM – revolutions per minute

RT – room temperature

RVR – rotovap residue

SID – siderophore

Sm – streptomycin

spp. – species

Tc – tetracycline

TCA – trichloroacetic acid

Tp - trimethoprim

TSA – trypic soy agar

µg – microgram

µl – microlitre

µM – micromolar

1. INTRODUCTION

1.1 *Burkholderia pseudomallei* and Melioidosis

In 1912, Whitmore and Krishnaswami published "An account of the discovery of a hitherto undescribed infective disease occurring among the population of Rangoon"[1] in which they described a human like glanders condition among morphine addicts and street people. They isolated a motile bacillus that demonstrated bipolar staining and wrinkled colony morphology on agar. Today that organism has been classified as *Burkholderia pseudomallei*, and the spectrum of diseases it causes are grouped together as melioidosis.

1.1.1 Melioidosis

Burkholderia pseudomallei is a gram negative saprophytic bacillus that can be isolated from soil and stagnant water such as found in the rice paddies of endemic areas. Infection occurs via inhalation or ingestion of this pathogen, or by direct inoculation of skin abrasions through contact with contaminated water or soil.[2] This organism is endemic to Southeast Asia and Northern Australia and is generally found between 20 degrees north and south latitudes.[3] Rice paddy farmers are particularly at risk due to their continued contact with stagnant water. Another group that was endangered was the military personnel stationed in Vietnam during the Vietnam War. It is estimated that one third of all military melioidosis cases were helicopter crewmen, and it is hypothesized that those who developed pulmonary melioidosis did so via inhalation of the soil organism raised by the helicopter's rotors.[3] Subclinical melioidosis is wide spread with 29% of the indigenous population[4] and 1.1% of the American soldiers (average stay of six months)[5] demonstrating positive hemagglutination reactions.

Melioidosis has been dubbed 'The Remarkable Imitator' due to the variability in its clinical presentation.[6] Melioidosis can mimic pulmonary tuberculosis, bacterial pneumonias or generalized sepsis.[6] This has resulted in diagnostic problems and inappropriate treatments. Melioidosis has a variety of clinical manifestations that can be categorized as acute, subacute, chronic or latent illnesses.[2] Melioidosis can present as an inapparent infection, an asymptomatic pulmonary infiltration, an acute localized suppurative infection, an acute pulmonary infection, an acute septicemic infection or a chronic suppurative infection.[2] It is frequently characterized by pulmonary infiltration, cystic cavities, pleural effusion, empyema, cough and tracheobronchitis.[6] The incubation period of this disease has not been established. It ranges from 2 days to 29 years in latent infections.[2,7,8] The longest recorded dormant period is in the case of a Vietnam veteran who had a reactivation of melioidosis after an inapparent infection acquired in an endemic area 29 years earlier.[8]

Diabetes mellitus is a predisposing risk factor for acute septicemic infections.[2] Other risk factors for infection and relapse include blast injuries, burns, malignancies, steroid treated systemic lupus erythematosus, renal failure, cancer chemotherapy, intravenous drug use and acquired immunodeficiency syndrome.[8]

The mortality rate of septicemic melioidosis is 90-95%. However, this drops to 50% if treated with appropriate antibiotic therapy.[3,9,10] *B. pseudomallei* is intrinsically resistant to a variety of antibiotics including beta lactams, aminoglycosides, macrolides and polymyxins.[3] Antibiotic therapy today involves new generation beta-lactams such as ceftazidime.[10] Treatment with 120mg/kg/day has lowered mortality by 50% although the optimal duration for the course of treatment has yet to be determined.[10] Current dogma is

that parenteral antimicrobial therapy should be given for a minimum of 10-14 days and continued until there is evident symptomatic improvement.[11] Maintenance antibiotic treatment is required, as relapse is a concern. Even with four months treatment, 10% of patients relapse.[12] However, due to the expense of antibiotic therapy, prolonged treatment is not always feasible and relapse continues to be a concern.

Human-to-human transmission of *B. pseudomallei* is rare but cases have been reported. In one case a Vietnam veteran, culture positive for *B. pseudomallei*, was shown to have infected his wife. Although her cervival and vaginal samples were culture negative, her increased serum hemagglutination titres was seen to be indicative of recent infection.[13]

Laboratory acquired melioidosis is also rare but not unheard of. A bacteriologist, at the University of Toronto, acquired melioidosis through a combination of inoculation and inhalation after a leak developed in a centrifuge.[14] In New York, a man was infected while sonicating what he believed to be *Burkholderia cepacia*.[15] After onset of disease, the organism was re-tested and revealed to be *B. pseudomallei*. The same organism was isolated from swelling in the patient's body.

1.1.2 General Pathogenesis of *Burkholderia pseudomallei*

B. pseudomallei can be isolated using Ashdown's medium[16] which is both selective and differential. Selectivity is based on *B. pseudomallei's* resistance to crystal violet at 5mg/l and gentamicin at 4mg/l. Differentiation is due to the ability of *B. pseudomallei* colonies to take up neutral red and demonstrate purple colony morphology. Ashdown's media can differentiate between *B. pseudomallei* and the closely related *B. thailandensis*, which is pink. Originally thought to be less virulent and *B. pseudomallei*-

like, this strain has been reclassified as *Burkholderia thailandensis*[17,18] based on genotypic and phenotypic differences. Sequence comparison of the 16S ribosomal RNA genes demonstrated a 15 nucleotide dissimilarity between the two strains.[18] These two strains also have different virulence in animals,[19,20] secretion profiles (lipase, lecthinase, siderophore, protease and cytotoxic acitivity of supernatant antigens)[17], LPS profiles[17], colony morphologies[17] and abilities to assimilate arabinose.[21]

B. pseudomallei is motile by means of a polar tuft of flagella that can be visualized by electron microscopy.[22] A flagellin monomer with a Mr of 43400 Da has been isolated and used to raise polyclonal antibodies.[23] These antibodies have been shown to specifically inhibit the motility of *B. pseudomallei* and passively protect diabetic rats. However, motility mutants do not demonstrate lower virulence in animal models suggesting that flagella is not a virulence determinant.[24]

B. pseudomallei is nutritionally diverse and can grow at temperatures from 5 to 42 degrees Celsius.[9] Optimal growth occurs between pH 5 and pH 8.[25] It has been shown to survive up to three years in sterile distilled water at ambient temperature as demonstrated by viable colony counts.[26] It has a high Guanine + Cytosine content, 67.9-69.5%[27,28], and has recently been ribotyped by restriction length polymorphism of rRNA genes.[29,30] Ribotyping has demonstrated that clinical *B. pseudomallei* isolates can also be found in the environment but with a markedly different RFLP pattern from other environmental isolates.[31] RAPD now allows subdivision of strains from the same ribotype.[32]

This pathogen produces several putative virulence factors including lipopolysaccharide/endotoxin[33] and exotoxin.[34,35] A metalloenzyme demonstrating alkaline protease activity was isolated from *B. pseudomallei* culture supernatants.[36] A

mutant, singly deficient in PRT activity demonstrated decreased virulence compared to the parent strains, suggesting that PRT may be a significant virulence factor. The siderophore produced by *B. pseudomallei* is of the hydroxymate class and has demonstrated the ability to scavenge iron from transferrin.[37] It may play a role in pathogenensis, allowing *B. pseudomallei* to survive and multiply in vivo. This pathogen also secretes a lipase[38] and a lecithinase (a heat labile phospholipase C).[38,39] In one study of 100 clinical isolates, over 90% secreted a LET, LIP, PRT and weakly cytolytic hemolysin into the extracellular milieu.[38]

Burkholderia pseudomallei has two separate LPS moieties that differ in the chemical structure of the O-antigenic polysaccharide.[40,41] LPS Type I has an O-antigen composed of 2-O acetyl-6-deoxy manno-heptose repeats. The O-antigen of LPS Type II is a D-glucose β-1,3-6 deoxy-L-talose repeat. O-PS II is a requisite for this pathogen's resistance to normal human serum. Serum sensitive mutants that were deficient in the O-PS II moiety, demonstrated increased sensitivity to killing by the alternative complement pathway and less virulence in animal models compared to the parent strain.[42] Analysis of the flagellin and O-PS have shown high degrees of conservation.[23,40,41] A conjugate vaccine incorporating the O-PS antigens and the flagellin protein linked by adipic acid dihydrazide elicited a high IgG response that protected diabetic rats from *B. pseudomallei* challenge.[43]

An exopolysaccharide was identified and isolated from a mucoid strain of *B. pseudomallei*.[44] A monoclonal antibody raised to the EPS was recognized by geographically diverse samples of *B. pseudomallei*. This EPS is an unbranched polymer

of 3 D-galactose residues and a 3-deoxy-D-mann-octulosonic acid with an O-acetyl group at the 2 position.[45,46]

The mechanism of secretion of certain exoproducts has recently been elucidated.[47,48] Mutations resulting in the inability to secrete lipase, lecithinase and protease have been mapped and were found to cluster at a locus with homology to the general secretory (type II) secretion pathway.

1.1.3 Intracellular Pathogenesis of *Burkholderia pseudomallei*

One interesting observation in the clinical progression of melioidosis is the recrudescence of the disease. There are reported cases of the reactivation of latent *B. pseudomallei* infections into acute and fulminating diseases at 26 and 29 years after initial infection.[7,8] Ribotype analysis has demonstrated that relapse can result from a persistent source of endogenous infection.[29,30] Relapse of infection when the course of treatment is not long enough, as well as difficulty in antimicrobial therapy despite *in vitro* susceptibility, suggest that invasion and survival in the intracellular environment are important factors in the pathogenesis of chronic and latent melioidosis.[12]

Invasion of eukaryotic cells is the first step in a successful endogenous infection. *B. pseudomallei* has been shown to successfully invade a variety of eukaryotic cell types, including cultured epithelial cell lines, such as HeLa, CHO, A549, Vero, CaCO-2, Hep2, L929 and McCoy and professional phagocytic cells such as rat macrophages and human polymorphonuclear neutrophils.[49,50] Electron microscopic visualization of infected HeLa cells, PMNs and U937 monocytes confirmed the presence of intracellular bacteria localized in membrane bound vacuoles. These vacuoles can contain either individual or multiple bacteria.[49]

Although little is known about the genetics of invasion, the irlRS locus has been shown to be an essential component. The irlRS locus encodes proteins that are transcriptional activators of a two component regulatory system in which signal transduction is accomplished by a phosphotransferase system. A strain deficient in this signaling system has demonstrated decreased invasive abilities as well as increased sensitivity to cadmium and zinc. However, in models of acute infection, the parent and mutants strains demonstrated comparable virulence suggesting that the irlRS locus is probably not a virulence determinant in acute infections.[51]

Little is known about the host response to challenge by *B. pseudomallei*. Studies have suggested an obligatory role for gamma interferon in the host survival of Taylor Outbred mice.[52] In this study, neutralization of IFN-γ, tumor necrosis factor or interleukin-12 increased susceptibility to infection *in vivo*. *B. pseudomallei* was shown to replicate focally in splenic abscesses without causing systemic toxicity, a necessary feature for chronic and latent infections. This confirms earlier observations that IFN-γ stimulated macrophages were more effective in *B. pseudomallei* killing than unstimulated macrophages.[53]

B. pseudomallei has the capability to replicate in PMNs over 20 hours. Incubation with normal human sera (NHS) did not affect uptake or intracellular survival of this pathogen.[49] In fact, *B. pseudomallei* is resistant to the bactericidal action of NHS[54] and can even multiply in 10-30% NHS.[42] As infection usually occurs via inhalation or inoculation of this pathogen, one of the first non specific host defense mechanisms encountered will be NHS in the circulation. The ability to resist bactericidal killing of NHS is therefore of great advantage to *B. pseudomallei*.

Numerous studies have demonstrated that this pathogen can survive and replicate in the intracellular environment of macrophages[55,56] and PMNs.[57] By electron microscopy, using an isolate of *B. pseudomallei* sensitive to gentamicin, it was shown that non-opsonized and mouse serum opsonized bacteria were internalized by RAW 264, U937 and HeLa cells and maintained in membrane bound vacuoles which disintegrated after time, allowing the bacteria to contact the host cytoplasm.[58] Despite EM examinations of infected RAW 264 macrophages, no evidence of a phagosome-lysosome fusion was found. Additional studies, using this unique gentamicin sensitive strain, have demonstrated the intracellular replication of *B. pseudomallei* in epithelial cells and fibroblasts. After internalization, the formation of multinucleate giant cells or syncytia was seen in nine of twelve eukaryotic cell lines (macrophage-like, epithelial and fibroblast).[50] This novel cytopathic effect was seen in tissue cultures infected by *B. thailandensis* and *B. mallei* as well.

By EM analysis it was shown that *B. pseudomallei* produces an EPS glycocalyx in the lung tissue of infected humans and animals, and it was hypothesized that the EPS may prevent bacterial opsonization and phagocytosis.[59] More recently it has been shown that *B. pseudomallei* activates complement, resulting in the deposition of opsonically active C3b and iC3b fragments of it surface via the alternative pathway.[60] Despite the fact that phagocytosis of this pathogen by polymorphonuclear leukocytes was significantly increased by opsonization, there was no significant bacterial killing noted. *B. pseudomallei* was also shown to be resistant to protamine, a cationic peptide, and to human defensin HNP-1 which may constitute a mechanism of intracellular survival.[49]

1.2 Bacterial Invasion and Intracellular Survival

Entry into and survival in host cells is a strategy unique to a certain subset of pathogens. It confers many advantages on the pathogen such as avoidance of host immune system and localization in a nutrient rich environment. However, there are obstacles as well. An intracellular lifestyle requires the abilities to survive the intrinsic defenses of the host cell as well as to multiply and, frequently, escape.

1.2.1 Bacterial Invasion

The fecal-oral route of transmission is common in some invasive pathogens such *Shigella, Salmonella* and *Yersinia.* After surviving the low pH environment of the stomach, they invade the mucosal epithelium of the colon, usually by induced endocytosis which requires exploitation of host microfilaments.

The mechanism of *Shigella* invasion involves localized membrane ruffling (via polymerization of actin and myosin) which results in pseudopod extension and bacterial engulfment.[61] Internalized bacteria are found within membrane bound vacuoles that are lysed soon after entry, releasing the pathogens to the host cytoplasm.[62] Escape requires a contact hemolysin that is plasmid encoded and essential to intracellular replication.[62] After bacterial replication and inhibition of host protein synthesis, the host cell is lysed allowing the progeny *Shigella* to infect neighboring cells.

Several *Shigella* invasion determinants have been localized to plasmid encoded loci. Included on this plasmid are the *ipa* (invasion plasmid antigen) genes, whose products are necessary to bind eukaryotic cell surfaces.[63] These genes are positively regulated by *virF,* which is essential to invasion.[64] Three Ipa proteins, IpaB, IpaC and IpaD, have been shown to bind $\alpha_5\beta_1$ integrins in CHO cells.[65]

Salmonella typhimurium also invades the intestinal epithelium. However, this pathogen continues to deeper tissues and the reticuloendothelial cells. *Salmonella* invasion causes the loss of apical epithelial microvilli and disrupts tight junctions, before invading and residing in membrane bound vacuoles that transcytose to the opposite cell surface. Invasion occurs via a membrane ruffling mechanism in which the host cell surface extrudes outward by way of actin/mysoin rearrangement.[66]

Salmonella typhimurium also harbors a plasmid essential for intracellular survival but not invasion. Multiple genetic loci involved in invasion are clustered on a pathogenicity island, SPI1, found in the chromosome.[67] The *inv/spa* gene cluster in SPI1 encodes one protein that is homologous to LcrD from *Yersinia* spp. and MxiA from *Shigella flexneri.*[68] This locus is believed to mediate the formation of a cell surface organelle necessary for invasion.

Yersinia pseudotuberculosis and *Yersinia enterocolitica* invade the Peyer's patches in the small bowel, where they transcytose to the reticuloendothelial system and eventually the lymph nodes and spleen.[69] Once internalized, this pathogen also resides in membrane bound vacuoles.

As with the other invasive pathogens, *Yersinia* spp. harbor a plasmid necessary for intracellular multiplication but not invasion or intracellular survival. The *inv* (invasion) gene is chromosomal and encodes a single cell surface polypeptide called invasin.[70] Without this gene *Y. pseudotuberculosis* is unable to adhere or invade tissue culture cells. Invasin tightly binds a family of host β_1 integrins and mediates bacterial uptake via a zipper-like mechanism in which the host cell membrane zippers around the pathogen.[71] This process utilizes host actin. *Y. enterocoliticia* also contain an *ail*

(attachment invasion locus) locus, which encodes a membrane protein required for strong adherence to epithelial cells and serum resistance.[72] Pathogenic *Yersinia* spp. also contain the Yad A plasmid encoded protein which binds host β_1 integrins.[73]

Shigella, Salmonella and *Yersinia* all appear to require the exploitation of host actin and myosin (microfilaments) to successfully invade non professional phagocytes, as internalization can be blocked by inhibition of host microfilaments. Internalization is not dependent on the rearrangement of host tubulin (microtubules). However, other pathogens such as EPEC, EHEC, *N. gonorrhoeae* and *K. pneumonia* do require the exploitation of host microtubules for successful invasion. Organisms that require host microtubules for invasion are usually not those pathogens for which invasion is an essential virulence mechanism.[74]

1.2.2 Bacterial Intracellular Survival

There are several mechanisms that intracellular pathogens have developed to evade the host immune response. These include invading non professional phagocytes, escaping to the host cell cytoplasm, interference with reactive oxygen intermediates, inhibition of phagosome-lysosome fusion; inhibition of the phagosome-lysosome acidification and resistance to lysosomal contents or cationic peptides such as defensins. Bacterial capsules and LPS provide a protective envelope around the bacteria, while certain bacterial enzymes can neutralize oxygen radical or degrade host lysosomal proteins.

Shigella flexneri dissolves the vacuolar membrane and gains access to the nutrient rich cytoplasm. Release from the vacuole is essential for replication and finally escape from the host cell. The IpaB gene product mediates escape from the endocytic vacuole.

VirF, which mediates invasion, also regulates virG, essential for the spread of intracellular bacteria to adjacent cells.[64]

Salmonella spp. reside in vacuoles in professional and non-professional phagocytes. *S. typhimurium* requires the acidic pH found post phagosome-lysosome fusion for the synthesis of the *pagA* gene products (required for intracellular survival) and to initiate intracellular replication.[75] The *Salmonella* containing vacuole lacks several of the normally delivered lysosomal markers, indicating the occurrence of an incomplete fusion.[76] Several factors enhance *Salmonella* intracellular survival, including the PhoP/PhoQ two component regulatory system that activates (pag) and represses (prg) bacterial gene products.[77] This system regulates resistance to bactericidal cationic peptides and inhibits antigen processing and presentation by host cells.[78]

Mycobacteria tuberculosis modifies the primary phagosome to prevent acidification and then prevents fusion with lysosomes. In the case of *Yersinia*, phagosome-lysosome fusion and degranulation occur. However, there is a decreased oxidative burst, allowing *Yersinia* survival

1.2.3 Phagosome-Lysosome Survival

Lysosomes are cytoplasmic organelles with a lipoprotein membrane that contain a myriad of different enzymes, mostly acid hydrolases.[79] The optimal activity of these enzymes is at an acid pH and they are collectively capable of degrading virtually all cellular molecules. The major lysosomal proteinases are cathepsins or thiol proteinases. Lysosomes are 50 – 100 nanometers in diameter and found in virtually all mammalian cells (except erythrocytes). The pH of the lysosome is believed to be between three and five. Lysosomes go through different stages. An endosome is prelysosomal and does not

possess a full range of lysosomal enzymes. Primary lysosomes contain a full armament of enzymes but have yet to be used for intracellular digestion and secondary lysosomes occur once a phagosome has fused with the lysosome.

Pathogens have evolved various means to avoid or overcome lysosomal attack.[79] *Coxiella* grows best at low pH, and *Leishmania* takes up amino acids and glucose better at acid pH. *Yersinia pestis* and *Mycobacterium lepraemurium* replicate in the phago-lysosome. Conversely, *Mycobacterium avium* inhibits the phagosome-lysosome fusion. *Listeria monocytogenes* and *Shigella spp.* lyse the membrane and escape to the host cell cytosol. *Salmonella typhimurium* reside in macrophages and epithelial cell phagosomes with some lysosomal markers indicating a partial phagosome-lysosome fusion. Lysosomal membrane glycoproteins are present but not certain lysosomal enzymes such as cathepsin D, leading to the hypothesis that these glycoproteins could protect the phagosome from the hydrolytic enzymes present in the lumen of the lysosome.[76] *Legionella pneumophila* modifies the pH of its phagocytic vacuole which prevents phagosome-lysosome fusion.[80]

1.3 Bacterial Exopolysacharides

1.3.1 Function

Most bacteria produce cell associated or secreted polysaccharides that can act as antigens or protective factors. Bacterial polysaccharides include capsules, lipopolysaccharides, lipooligosaccharides and extracellular polysaccharides. These polymers vary considerably in their chemical structure, biosynthesis and regulation.

Bacterial polysaccharides are highly hydrated and can prevent desiccation.[81] This aids in the transmission of encapsulated bacteria from one host to the next. In some instances, desiccation is a cue for increased expression of genes encoding EPS production. *E. coli* upregulates production of colanic acid in response to desiccation. Current belief is that desicccation changes the external osmolarity which, in turn, is the trigger for increased EPS biosynthesis. *Pseudomonas aeruginosa* and *Salmonella typhi* both respond to high external osmolarity.

Bacterial polysaccharides may increase the adherence of bacteria to surfaces allowing colonization, the initial step to invasion and intracellular survival.[81] EPS may also increase adherence to other bacteria, facilitating biofilm formation. Biofilms may protect pathogens from phagocytosis or confer nutritional advantages. In *Pseudomonas aeruginosa* alginate rich biofilms may buffer the bacteria from antibiotics. Conversely, capsular polysaccharides in *Proteus mirabilis* facilitate swarming due to their lubricating properties.

Bacterial polysaccharides may aid in resistance to nonspecific host immunity by providing a barrier to complement mediated killing.[81] It may mask cell surface components that would activate the alternative complement pathway. Some polysaccharide are believed to be poorly immunogenic and thus aid in resistance to specific host immunity. Some are poorly immunogenic due to their structural similarity to host cell surface saccharides. These include polysaccharides produced by *E. coli* K1 and *N. meningitidis* serogroup B. The presence of these polysaccharides confer a clear advantage to the bacterial pathogen.

1.3.2 Environmental Stimuli

Alginate is, perhaps, the best studied bacterial exopolysaccharide.[82] Alginate is produced by a variety of microorgansims, including *Azobacter vinelandii* and *Pseudomonas aeruginosa.* It is a random polymer of β-1,4-linked D-mannuronic acid and its C5 epimer L-gluronic acid. It is highly acetylated which allows the retention of moisture in a dehydrated environment such as the cystic fibrosis lung. Alginate is also believed to protect this opportunistic pathogen from antibiotic treatment, phagocytosis and promote adherence to lung epithelium. One major signal for the transition to mucoidy is nutrient starvation, particularly phosphate or nitrogen.[83] This trigger enhances the gene expression of energy-yielding reactions in the tricarboxylic acid cycle and energy transduction. Studies have shown that iron limitation, a condition seen in the CF lung, also triggers conversion to mucoidy.[84] Growth under energy limiting conditions or on energy poor sources of carbon or nitrogen also results in conversion to mucoidy.[83] Clearly, the conversion to a mucoid phenotype confers an advantage in terms of energy demands on the bacterial cell.

It has been suggested that the mucoid form enhances the ability of *P. aeruginosa* to scavenge scarce nutrients due to ionic attraction to the negatively charged alginic acid polysaccharide.[84] Another advantage to the mucoid form may be the conservation of energy by recycling organic acids and creating an electrochemical gradient.[84] More recent studies have suggested that *AlgR2*, a regulatory gene in alginate biosynthesis, also maintains the proper activity of the TCA cycle through succinyl-CoA synthetase.[85] The TCA cycle functions in the terminal oxidation of nutrients and the generation of energy and reducing equivalents. AlgR2 also appears to regulate a nucleoside diphosphate

kinase, Ndk, which transfers the terminal phosphate from any NTP or dNTP to any other NDP or dNDP demonstrating the importance of nucleoside triphosphate synthesis and energy metabolism for alginate synthesis.[85] Perhaps it is the modulation of NTP pools by environmental conditions that may trigger alginate production.

There are many other bacterial EPS that respond to a variety of environmental stimuli. *Staphylococcus aureus* capsular polysaccharide type 5 is negatively regulated by the concentration of CO_2.[86] *Rhizobium meliloti* induces a succinoglycan in raised osmotic pressure[87] and a second, chemically distinct, EPS under low phosphate condition.[88] *E. coli* K1 capsule production is regulated by environmental temperature and assists in resistance to opsonization.[89] Enterohemorrhagic *E.coli* O157:H7 EPS production is upregulated by anaerobic conditions and growth at 37°C.[90] EPS production is a phenomenon that confers many advantages on certain bacterial pathogens. The induction of these polysaccharides is regulated by environmental stimuli, which allows their immediate response to susceptible hosts or to potentially bactericidal conditions.

1.4 Global Regulation of Bacterial Virulence Factors

Many virulence genes are coordinately regulated by a variety of environmental and genetic signals. *Salmonella typhimurium in vivo*-induced (*ivi*) genes were categorized by their coordinate behavior to low pH, low magnesium concentrations, iron limitation, as well as to the PhoPQ regulatory system.[91] These genes were also shown to display coordinate induction upon entry into cultured murine macrophages and human epithelial cells. Low pH and low Mg^{2+} concentrations are seen within the vacuoles that Salmonella reside in eukaryotic cells and low Mg^{2+} concentrations have also been shown

to induce the activation of the PhoPQ regulatory system. Iron limitation is a well-known obstacle to bacterial infection and would thus logically be an important environmental signal. The *ivi* genes respond to intracellular signals that are present in initial and progressive stages of infection.

The PhoPQ system governs the transcription of 25 loci in response to extracellular magnesium concentrations.[92] Included in these loci, is the *pmrCAB* loci, which encodes a two component regulatory system for polymyxin B resistance. The PmrA response regulator protein is induced by Mg^{2+} limitation and also by mild acidification. It is essential for the transcription of seven of PhoP-activated loci, allowing these loci to respond to wider variety of environmental signals.

The regulatory protein ToxT activates the transcription of the virulence factors in *Vibrio cholerae*, such as cholera toxin and the toxin coregulated pilus.[93] Transcriptional activation by the ToxT protein is modulated by environmental signals. Positive regulation occurs at 30°C but not 37°C. There is also negative regulation in the presence of 0.4% bile. Malnutrition is a predisposing factor to cholera. Since bile is released in response to food intake, low bile concentrations would result from malnutrition and signal *V. cholerae* that a susceptible environment for infection exists.

In *Bordetella pertussis*, global regulation of all virulence associated genes is by the transcriptional activator *vir* and in response to environmental cues.[94] *Vir* regulates the expression of pertussis toxin, adenylate cyclase toxin, filamentous hemagglutinin and dermonecrotic toxin. Modulation of *vir* is seen in the presence of sulphate ions, which eliminated transcription of *vir*-regulated genes, but not in the presence of Na^+, K^+, Mg^{2+},

NH^{4+} or Cl^-. A low growth temperature of 25°C reduces transcription of *vir*-regulated genes. Perhaps modulation of the *vir*-regulated genes allows this pathogen to exist without damaging the host. At optimal times, when the modulating signal has been removed, the organism can respond to a susceptible host by the rapid activation of the *vir*-regulated genes.

1.5 Hypothesis and Objectives

It is hypothesized that both the ability to invade and the ability to survive in the intracellular environment of eukaryotic cells are critical to the pathogenesis of melioidosis, particularly in the case of disease recrudescence. The production of EPS is believed to play a necessary role in intracellular survival. To test our hypothesis, we formulated a number of objectives for our studies. These were:

1. To study the invasion of epithelial A549 cells by *B. pseudomallei* and to identify the genes involved in invasion.
2. To study the intracellular survival of *B. pseudomallei* in the epithelial A549 cell line by observing replication rates and compartmentalization. Specifically, we investigated the inter-vacuolar environment, to ascertain whether there is a phagosome-lysosome fusion.
3. To determine if resistance to low pH is necessary for intracellular survival and to identify a putative exopolysaccharide that *B. pseudomallei* produces in response to acidic conditions.

2. MATERIALS AND METHODS

Table 1. **Bacterial strains and plasmids**

Strain or plasmid	Description	Source or Reference
Strains		
E. coli		
SM10	Mobilizing strain, transfer genes of RP4 Integrated in chromosome: Km^R, Sm^S	95
SM10λ	SM10 with a λ prophage carrying the gene encoding the π protein	96
DH5α	High-efficiency transformation. F⁻ϕ80d*lacZ ΔM15 Δ(lacZYA-argF)U169 EndA1 recA1 hsdR17 deoR thi-1 supE44 gyr A96 relA1*	BRL
HB101	F- Δ(gpt-proA)62 leu supE44 ara 14 galK2 lacY1 Δ(mcrC-mrr) rpsL20 (Sm^R) xyl-5 mtl-1 recA13	97
SURE	High-efficiency transformation; deficient in homologous recombination; e14;(mcrA)Δ (mcrCB-hsdSNR-mrr)177 endA1 supE44 thi-1gyr A96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC [F' proAB lacI9ZΔM15 Tn10]; Km^R, Tc^R	Stratagene
B. pseudomallei		
1026b	Clinical isolate; Sm^R, Tc^S, Tp^S	D.A.B. Dance
AJ1D8	1026b derivative; irlR::Tn5-OT182	51
1026b (pUCP28Tgfp1)	1026b (pUCP28T::gfp1); Tp^R, Sm^R	This study
B. thailandensis		
E264	Environmental isolate: Sm^R, Tc^S	D.A.B Dance
Plasmids		
pOT182	pSUP102(Gm)::Tn5-OT182; Cm^R, Gm^R, Ap^R, Tc^R	98
pUCP28T	Broad-host-range vector; IncP OriT; ColE1 ori; Ap^R, Tc^S	99
pKENgfp1	Ap^R	100

2.1 Bacterial and Tissue Culture Strains and Growth Conditions

The bacterial strains and plasmids used in these experiments are described in Table 1. All bacterial strains were maintained on Luria Bertani (LB) agar (Gibco Canada Inc., Missssauga, Ontario, Canada) or trypic soy agar (TSA) (Mikrobiologie, BDH, Germany) and grown in LB broth. When appropriate, antibiotics were added at the following concentrations: 100 μg/ml ampicillin (Ap), 25 μg/ml kanamycin (Km), 25 μg/ml chloramphenicol (Cm), 12.5 μg/ml tetracycline (Tc), 100 μg/ml streptomycin (Sm) and 1.5 mg/ml trimethoprim (Tp) for *E. coli*. For *B. pseudomallei*, 100μg/ml Sm, 50 μg/ml Tc, or 225 μg/ml Sm, 100 μg/ml Tp were added when appropriate.

Burkholderia pseudomallei and *Burkholderia thailandensis* isolates were generously provided by Dr. D.A.B. Dance, Wellcome-Mahidol University, Oxford Tropical Medicine Research Program, Bangkok, Thailand. *Salmonella typhmurium* 14028s was provided by Dr. K. Sanderson, Salmonella Genetic Stock Center, University of Calgary, Calgary, Alberta, Canada.

A549 type II pneumocytes were obtained from American Type Culture Collections (Rockville, Md.). A549 type II pneumocytes were maintained in F12K media (Gibco Canada Inc., Missssauga, Ontario, Canada) containing standard antibiotic mixture (0.25 μg amphotericin B, 100 U penicillin and 0.1 mg streptomycin per ml) (Sigma, St. Louis, Mo) plus 10% Fetal Bovine Serum (FBS) (Fetalclone, Hyclone, Logan, Utah) at 37°C with 5% CO_2.

2.2 Invasion and Intracellular Survival

2.2.1 Quantitative Invasion Assay[101]

A549 type II pneumocytes were seeded at 5 x 10^5 cells/well in F12K plus 10% FBS and grown overnight to confluency in 24 well plates (Falcon, Becton Dickinson, Franklin Lake, NJ). Overnight bacterial cultures were subcultured 1:30, grown for 4-5 hrs at 37°C, 250 RPM and then diluted to OD (600nm) 0.53-0.55. The eukaryotic monolayers were washed and, after the addition of 1ml F12K, infected with mid-exponential phase bacteria at a MOI=10. The monolayer was centrifuged for 10 minutes at 1000 RPM to facilitate infection. Serial dilutions of the inoculum were plated in order to quantitate initial bacterial load. After 2 hours infection at 37°C, 5% CO_2 , extracellular bacteria were killed with 225 μg/ml Km. The monolayer was lysed with 1% Triton X-100 (BDH Chemicals, Toronto, Ontario, Canada) after an additional 2 hours at 37°C, 5% CO_2 and plated in serial dilutions to quantitate the number of intracellular organisms. All invasion assays were performed in triplicate.

2.2.2 Inhibition of Invasion by Nocodazole [102,103]

Bacterial invasion may involve the exploitation of certain host cell cytoskeletal functions. To determine if the uptake of *B. pseudomallei* into cultured epithelial cells is dependent on host microtubule function, nocodazole (Sigma, St. Louis, Mo) was used to specifically depolymerize the microtubules and inhibit their function. A549 monolayers were preincubated with 10 μg/ml nocodazole (Sigma Chemical Co., St. Louis, Mo) in DMSO (Sigma Chemical Co., St. Louis, Mo) for 60 min. on ice then warmed to 37°C for 30 min. Invasion assays were performed as described except that nocodazole was

maintained in the media throughout the assay. Assays in which the eukaryotic monolayers were preincubated with DMSO or without drugs were performed as controls.

2.2.3 Transposon Mutagenesis and Generation of Invasion Deficient Mutants

Transposon mutagenesis of *B. pseudomallei* was performed using the Tn5-OT182 transposon.[24] *B. pseudomallei* 1026b and *E. coli* SM10(pOT182) were grown for 18 hours (37°C, 250 RPM) in LB broth and LB broth containing 25 μg/ml Km, 25 μg/ml Cm, and 12.5 μg/ml Tc respectively. The donor and recipient were mixed in 10 mM $MgSO_4$ and filtered through a 25 mm Swinnex filter apparatus (Milliore Corp., Bedford, MA) containing a 0.45 μm nitrocellulose filter. Control matings of solely the donor or recipient cultures were performed. The filters were incubated on LB agar plus 10 mM $MgSO_4$ for eight hours at 37°C. Bacteria were resuspended in 0.85% NaCl and plated on LB agar supplemented with 100 μg/ml Sm to select against the *E. coli* donor strain and 50 μg/ml Tc to select for the transposon insertion. Transconjugants were identified after 48 hours at 37°C.

Transposon mutants were screened for invasion deficiency by qualitative invasion assays.[51] A549 cells were grown to confluency in 96 well plates (Falcon, Becton Dickinson, Franklin Lake, NJ). Transposon mutants were isolated to 96 well plates (LB broth plus 50 μg/ml Tc) and grown overnight (37°C, 250 RPM). Mutants with approximately the same optical density (600nm) as the parent strain were used to infect individual monolayers. The plates were centrifuged for 10 min. at 1000 RPM to facilitate infection. Infection proceeded for 2 hrs (37°C, 5% CO_2) before extracellular bacteria were killed with 225 μg/ml Km. The monolayer was lysed with 1% Triton X-100 and

plated to LB agar plates plus 100 μg/ml Sm and 50 μg/ml Tc. After 48 hours at 37°C, invasion deficient mutants were recovered in lower numbers than the parent strain.

2.2.4 DNA Manipulation

Bacterial genomic DNA was isolated as previously described. [104]

DNA from *B. pseudomallei* invasion mutants was self cloned. Isolated bacterial genomic DNA was digested with restriction enzymes (Gibco/BRL, Bethesda Research Laboratories, Gaithersburg, Md or New England Biolabs, Beverly, Mass.) and ligated overnight at 16°C with T4 DNA ligase (Gibco/BRL, Bethesda Research Laboratories, Gaithersburg, Md). The ligation reaction was either chemically transformed or electroporated into competent *E. coli* DH5α using a GenePulser II/Pulse controler plus apparatus (BioRad, Richmond, CA). If necessary, the ligation mixture was concentrated using Microcon-YM 30 micro concentrators (Millipore Corp., Bedford, MA).

Plasmid DNA was isolated from overnight cultures using Wizard Plus Minipreps (Promega, Madison, WI) or QIAprep Spin Miniprep (Qiagen, Mississauga, ON). Plasmid DNA was digested with appropriate restriction enzymes and examined on a 0.8% ultra pure agarose (Gibco/BRL, Bethesda Research Laboratories, Gaithersburg, Md) gel. If necessary, individual bands were excised and cleaned using The Geneclean II Kit (Bio/Can Scientific, Mississauga, Ontario, Canada).

2.2.5 DNA Sequencing and Analysis

Automated sequencing of the DNA flanking the transposon insertion was performed by the University Core DNA Services (University of Calgary) using an ABI PRISM DyeDeoxy Termination Cycle Sequencing System and AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ). Oligodeoxyribonucleotide primers

previously synthesized to the right and left ends of the pOT182 transposon were used to facilitate these reactions. OT182-Lt (5'-GATCCTGGAAAACGGG AAAG-3') initiated DNA sequence reactions for plasmids obtained from self-cloning of the Tn5-OT182 invasion deficient mutants with *Sal*I, *Apa*I, *Stu*I, *Hind*III, *Not*I and *Xho*I. OT182-Rt (5'-ACATGGAAGTCAGATCCTGG-3') initiated DNA sequence reactions for plasmids obtained from self-cloning of the Tn5-OT182 invasion deficient mutants with *Sca*I, *Eco*RI, *Sst*I, *Cla*I and *Bam*HI.

DNA sequences were analyzed for homologous sequences using the blastx program[105] to search the non-redundant sequence database. Protein sequence alignment was performed using the Swiss Prot annotated protein sequence database provided by the ExPASy molecular biology server.

2.2.6 Intracellular Survival Assays[106]

Invasion assays were performed as described with the following exceptions. Following the killing of all extracellular bacteria with 225 μg/ml Km, the monolayer was washed with PBS and fresh F12K plus 75 μg/ml Km added. At desired time points, the monolayers were washed, lysed and the number of intracellular bacteria quantitated.

2.2.7 Electron Microscopy Analysis of *B. pseudomallei* infected A549 cells

Intracellular survival assays were performed as described. *B. pseudomallei* infected A549 cells were prepared for EM analysis as follows.[107] Infected monolayers were washed three times in 0.1M phosphate-buffered saline pH 7.2, fixed in 2.5% phosphate-buffered glutaraldehyde (EM Sciences, Fort Washington, Pa.) for 1 hour and post-fixed in 2% buffered osmium tetroxide (EM Sciences, Fort Washington, Pa.) for 1 hour at RT in the tissue culture wells. The monolayer was then washed twice with water,

gently scraped, aspirated, centrifuged and enrobed in 2% noble agar (Gibco/BRL, Gaithersburg, Md). The cells were stained with 1% uranyl acetate (Fisher Scientific, Fairlawn, NJ), dehydrated using a graded ethanol series and embedded in LR white medium grade resin (EM Sciences, Fort Washington, Pa.). The polymerized blocks were thin sectioned, the sections mounted on Formvar, carbon-coated, 400 mesh, copper grids (EM Sciences, Fort Washington, Pa) and stained with uranyl acetate and lead citrate (EM Sciences, Fort Washington, Pa). The sections were viewed in a Philips EM 300 operating at 60 kV under standard conditions. Electron microscopy was performed at the STEM Facility at the University of Guelph, Guelph, Ontario, Canada.

2.2.8 Construction of *B. pseudomallei* 1026b(pUCP28Tgfp1)

E. coli SURE (pKENgfp1) was graciously donated by Brendan Cormack (Stanford, California). The green fluorescent protein is expressed from a TAC promoter in the vector pKEN. The gfp1 sequence was cloned into pUCP28T as a Xba1 - Pst1 fragment. The plasmid was then electroporated into *E. coli* SM10λpir and conjugated with *B. pseudomallei* 1026b. *B. pseudomallei* 1026b (pUCP28Tgfp1) transconjugants were selected on 100 μg/ml Tm and 225 μg/ml Sm. The invasion of *B. pseudomallei* 1026b (pUCP28Tgfp1) in A549 cells was examined to ensure it was comparable to that of *B. pseudomallei* 1026b.

2.2.9 Confocal Fluorescent Microscopy Analysis of Phagosome-Lysosome Fusion

Phagosome-lysosome fusion was investigated using confocal fluorescent microscopy to assess for colocalization of fluorescence. *B. pseudomallei* 1026b (pUCP28Tgfp1) emits a green fluorescence due to the presence of the GFP protein. The lysosomes were labeled using the LysoTracker Red DND-99 probe (Molecular Probes,

Eugene, OR) which is a fluorescent acidotropic dye that accumulated in the lysosome due to its low internal pH. As this probe is somewhat photosensitive, this assay was performed in as dark an environment as possible. Intracellular survival assays were performed as described, using *B. pseudomallei* 1026b (pUCP28Tgfp1) with the following exceptions. A549 cells were grown to confluency on glass coverslips in 24-well plates. Lysotracker Red (70 nM) was added to the eukaryotic monolayer at the time of infection. Intracellular survival proceeded overnight, the coverslips were washed with ethanol, mounted inverted on glycerol and colocalization of fluorescence was assayed by confocal microscopy. A differential interference contrast filter was used to view cellular structures.

2.3 *B. pseudomallei* Response to Acidic Conditions

2.3.1 Cadmium Sensitivity Assay

TSA plates were prepared containing 5 mM cadmium chloride (Sigma, St. Louis, Mo) and, if required, 50 µg/ml Tc. Overnight cultures of *B. pseudomallei* 1026b or AJ1D8 in LB or LB plus 50 µg/ml Tc respectively were adjusted to McFarlands Standard 0.5, and 100 µl was spread on the cadmium supplemented plates. The plates were incubated for 48 hours at 37°C, and resistance was determined as growth in the presence of 5 mM cadmium chloride.

2.3.2 Heavy Metal Cations Minimum Inhibitory Concentrations

Bacterial cultures were grown overnight (37°C, 250 RPM) in appropriately supplemented Mueller Hinton Broth (Becton Dickinson, Cockeysville Md), diluted to McFarland's Standard 0.5 and diluted again 1:10. These cultures were used to inoculate tubes contained two-fold serial dilutions of the cation being tested and a growth control

(containing no cation). The tubes were incubated stationary, overnight at 37°C and read for visible turbidity equating to growth. The MIC was determined as the lowest concentration that inhibits bacterial growth.

2.3.3 Evaluation of Bacterial Intracellular pH

All experiments involving acidic pH were performed in sodium citrate/HCl buffers[108] supplemented with 0.4% glucose and 0.02 M NH_4Cl. At pH 4.5 the buffer was prepared as follows: 70.65 ml 0.1 M disodium citrate was added to 29.35 ml 0.1 *N* HCl and 0.02 M ammonium chloride. The media was autoclaved, cooled and 0.4% glucose was added.

Evaluation of the intracellular pH of *B. pseudomallei* after challenge at a variety of pHs was undertaken using Oregon Green Dye 488 (Molecular Probes, Eugene, OR). This dye has a pKa of 4.7 and is pH sensitive; there is virtually no fluorescence at neutral pHs. Broth cultures of *B. pseudomallei* 1026b and AJ1D8 in LB broth or LB broth plus 50 μg/ml Tc respectively were centrifuged, washed with NaCitrate/HCl buffers (pH3, pH5 or pH7) three times, and resuspended in the appropriate NaCitrate/HCL buffer plus 10 μM Oregon Green Dye (dissolved in dimethylformamide). The cultures were incubated at 30°C with shaking, spotted on a glass microscope slide and viewed immediately for fluorescence. The percentage of bacterial cells demonstrating fluorescence was established. Percentages were calculated using the mean of 10 fields of view.

2.3.4 *B. pseudomallei* Survival at Acidic pH

Broth cultures of *B. pseudomallei* 1026b and AJ1D8 in LB broth or LB broth plus 50 μg/ml Tc respectively were centrifuged, washed with NaCitrate/HCl buffers (pH3, pH5 or pH7) three times, and resuspended in the appropriate NaCitrate/HCl buffer. The cultures were incubated at 30°C with shaking, and 100 μl aliquots were plated on TSA or TSA plus 50 μg/ml Tc respectively. The plates were assessed for viability after 48 hours incubation at 37°C.

B. pseudomallei 1026b survival at pH3 was quantitated. A 1.5 ml broth culture of *B. pseudomallei* 1026b in LB broth was centrifuged, washed with NaCitrate/HCl buffer pH 3 three times and resuspended in NaCitrate/HCL buffer pH 3. Plating 10-fold serial dilutions to TSA plates quantitated the control number of cfu/ml. The bacterial suspension was then incubated at 30°C with shaking and plated in 10-fold serial dilutions. After 48 hours at 37°C, the number of cfu/ml was assessed and compared as a percentage to the control group plated before incubation at pH 3.

2.3.5 Evaluation of Optimal pH for *B. pseudomallei* Growth

Broth culture of *B. pseudomallei* 1026b in LB broth was subcultured 1:250 into a graded series of NaCitrate/HCl buffer at decreasing pHs and incubated at 37°C, 250 RPM. After 24 hours, growth was assessed by optical density at 600nm.

2.3.6 Growth Curves

A overnight culture of *B. pseudomallei* 1026b in LB broth was subcultured 1:100 into pH 4.5 NaCitrate/HCl buffer. At 0 hours and at 48 hours (37°C, 250 RPM), optical density (600nm) was compared to cell numbers (established by plating serial 10-fold dilutions).

For growth curves, *B. pseudomallei* 1026b was grown overnight at 37°C, 250 RPM in LB broth and then subcultured in parallel to LB or pH 4.5 NaCitrate/HCl buffer. Optical density (600nm) and cell number (cfu/ml) were determined at appropriate time points.

2.3.7 Urease Assay

Bacto urea broth was prepared (Difco Manual, Detroit Michigan). Overnight *B. pseudomallei* 1026b cultures were subcultured 1:100 into either LB or pH 4.5 sodium citrate/HCl buffer and incubated overnight (250 RPM, 37°C). Tubes with 3 ml of urea broth were inoculated 1:10 with either supernatants or whole cell cultures and incubated overnight at 37°C. Urease production was identified by a cerise colour.

Inducible urease production was also examined. M9 media was prepared with the exception that urea was included as the sole nitrogen source instead of ammonium chloride. Urease was required to cleave the urea in order for bacterial growth. Overnight cultures of *B. pseudomallei* in LB or M9 (plus urea) were tested for the presence of urease using Bacto urea broth as described previously.

2.4. Identification and Characterization of the Exopolysaccharide produced by *B. pseudomallei* at acidic pH

2.4.1 Orcinol-Sulphuric Acid Assay for Reducing Sugars[109]

In this assay, concentrated acid is used to cause the hydrolysis of all glycosidic linkages in the polysaccharide. The subsequent dehydration product reacts with the orcinol (Fisher Scientific, Fairlawn, NJ) to give a coloured endpoint. Overnight *B. pseudomallei* 1026b cultures were subcultured 1:100 into LB or pH 4.5 sodium citrate/HCl buffer and incubated overnight (250 RPM, 37°C). Whole cell overnight cultures or filter sterilized supernatant (200 µl) were cooled at 4°C and mixed with 800 µL freshly dissolved orcinol in sulphuric acid (2 g/l). The solution was heated at 80°C for 15 minutes, then cooled rapidly to RT. The absorbance was determined at 405nm. In order to quantitate the amount of exopolysaccharide produced, a standard curve of doubling dilutions of dextran sulphate (Sigma, St. Louis, Mo) (2 mg/ml) was assayed concurrently with the samples.

2.4.2 Enzyme Linked Immunosorbant Assays

B. pseudomallei 1026b cultures were subcultured 1:100 into either LB or pH 4.5 sodium citrate/HCl buffer and incubated for 18 to 24 hours at 250 RPM, 37°C. The bacterial culture was diluted in coating buffer, and 100 µl aliquots were used to coat wells in a 96-well plate (Nunc, Denmark) for 2 hrs at 37°C. The wells were washed twice with PBS-Tween (0.05% Tween 20 in 10 mM PBS) and blocked with 3% skim milk in PBS-T for 2 hrs at 37°C. The wells were again washed twice with PBS-T and incubated with the primary antibody for 2 hrs at 37°C. The wells were washed twice

with PBS-T and incubated with the secondary antibody for 2 hrs at 37°C. The wells were washed three times with PBS-T, developed at RT for 30 min and reaction assessed at 405nm.

To assess the presence of capsule, monoclonal antibody IgG 3015 (provided by Dr. I. Steinmetz, Germany) was used as the primary antibody at a 1:100 dilution. The secondary antibody was goat α mouse IgG•HRP (Sigma, St. Louis, Mo).

To assess the presence of LPS type I O-antigen and LPS type II O-antigen, all samples were boiled first to ensure the adherence of the LPS to the 96-well plate. LPS type I O-antigen was assessed using a polyclonal antibody that had been developed against LPS and flagella. Antibodies to the LPS type II O-antigen were absorbed out using *B. thailandensis* resulting in a polyclonal antibody to LPS type I O-antigen and flagella. Goat α rabbit IgG•HRP (Sigma, St. Louis, Mo) was used as the secondary antibody. LPS type II O-antigen was assessed using a monoclonal antibody developed to the LPS type II moiety (1:100) as the primary antibody and goat α mouse IgM•HRP (Sigma, St. Louis, Mo) as the secondary antibody.[110]

2.4.3 EPS Purification Protocol

The protocol used to isolate the EPS produced by *B. pseudomallei* 1026b at pH 4.5 is outlined schematically in Figure 17.[111] LB broth was inoculated with *B. pseudomallei* 1026b, grown for 18 hrs (37°C, 250 RPM), subcultured 1:100 into pH 4.5 NaCitrate/HCl buffer and grown for 36 hrs (37°C, 250 RPM). The bacterial culture was centrifuged for 15 min. at 16300 RCF, 4°C, and the supernatant was kept for further isolation. The pellet was resuspended in a minimal volume of cold 0.85% NaCl, stirred vigorously for 1 hr at RT in a fume hood, centrifuged for 15 min. at 16300 RCF, 4°C and

the supernatant combined with those previously isolated. At -6°C, 10% (w/v) sodium acetate and 1% (v/v) glacial acetic acid were added to the supernatant and stirred. Two volumes of cold 95% ethanol was added slowly, and the solution was stirred for 18 hours. The precipitate was removed by centrifugation (30 min. at 16300 RCF, 4°C). This resulted in two separate fractions, the ethanol precipitate and the ethanol supernatant.

The ethanol precipitate was rinsed with cold sterile water, resuspended in a minimal volume of cold 0.85% NaCl, dialyzed (Spectra/Por membrane 3, MWCO 3500, Spectrum, Gardena, Ca) against running water for 3 days and lyophilized. The proteins were removed by TCA precipitation, as described below, and then the precipitate was treated with DNase I (Sigma, St. Louis, Mo) and RNase A. (Sigma, St. Louis, Mo). The precipitate was run through a 105 centimeter sepharose CL-4B (Sigma, St. Louis, Mo) size exclusion column in 0.1 *M* phosphate buffer pH 7.2. The column was run at 1 ml/min., and 3 ml fractions were collected by a Pharmacia LKD RediFrac fraction collector after a 150 ml void volume (negative orcinol reaction for presence of polysaccharide). Fractions were analyzed using the orcinol-sulphuric acid assay, peaks were collected, dialyzed and lyophilized. These fractions are referred to as "EP" fractions.

In the ethanol supernatant fraction, the ethanol was evaporated off, and the remaining viscous solution was dialyzed against running water for 3 days and lyophilized. The proteins were removed by TCA precipitation, as described below. The ethanol residue was run through a 105 centimeter sepharose CL-4B size exclusion column in 0.1 *M* phosphate buffer pH 7.2. The column was run at 1 ml/min, and 3 ml fractions were collected after a 150 ml void volume volume (negative orcinol reaction for

presence of polysaccharide). Fractions were analyzed using the orcinol-sulphuric acid assay, peaks were collected, dialyzed and lyophilized. These fractions are referred to as "RVR" fractions.

Proteins were precipitated out of the isolated EPS using trichloroacetic acid (Fischer Scientific, Fairlawn, NJ). The EPS was dissolved at 20-40 ng/ml in sterile distilled water. Cold 50% TCA was added dropwise to a final concentration of 10% TCA while the solution was stirring in an ice-water bath. The solution was allowed to stand for 1 hr at 4°C and then centrifuged for 1 hr at 16300 RCF, 4°C. The supernatant was tested for the presence of any residual protein using the BioRad Protein assay (BioRad Lab, Richmond, Ca) and then dialyzed and lyophilized.

2.4.4 Glucose Assay

Residual glucose from the NaCitrate/HCl buffer was assessed using the Glucose Trinder Assay (Dcl, Biopacific Diagnostic Inc, Vancouver, BC). In this assay glucose is degraded to D-gluconic acid and H_2O_2 in the presence of glucose oxidase. H_2O_2 reacts with the glucose colour reagent and results in a coloured endpoint. Bacterial cultures were prepared as for the orcinol-sulphuric acid assay, 25 µl of sample were mixed with 2.5 ml of the supplied colour reagent and incubated at 37°C for 5 min. The absorbance was determined at 505 nm. In order to quantitate the amount of residual glucose in the media, a standard curve, prepared using glucose (6.25%) doubling dilutions, was assayed concurrently with the samples.

2.4.5 KDO Assay[112]

Purified EPS fractions were assayed for the presence of 3-deoxy-D-mannoctulosonic acid. In 0.2 *N* H_2SO_4, 40 ug of each fraction or standard was dissolved,

heated at 100°C for 30 min., centrifuged, and the supernatants were assayed. To the supernatants, 0.25 ml of 0.04 *M* H_5IO_6 (Aldrich Chemical Co, Milwaukee, Wis) in 0.125 *M* H_2SO_4 was added and mixed well. The mixture was allowed to stand at RT for 20 min. before 0.25 ml 2.6% $NaAsO_2$ (Sigma, St. Louis, Mo) in 0.5 *N* HCl was added and vortexed. Once the brown colour had disappeared, 0.5 ml of 0.6% thiobarbituric acid (Sigma, St. Louis, Mo) was added and vortexed. The reaction was heated at 100°C for 15 min. and 1 ml DMSO (Sigma, St. Louis, Mo) was added. After cooling to RT, the absorbance was read at OD 548nm. Pure KDO (Sigma, St. Louis Mo) was analyzed as a positive control.

2.4.6 Sialic Acid Assay[113]

Purified EPS fractions were assayed for the presence of sialic acid. In sterile water, 40 ug of each fraction or standard were dissolved and treated with 0.25 ml of *25 mM* H_5IO_6 in 0.125 *M* H_2SO_4 at 37°C for 30 min. The excess periodate was reduced with 0.2 ml 2% $NaAsO_2$ in 0.5 *N* HCl. Once the yellow colour had disappeared, 2 ml of 0.1 *M* thiobarbituric acid was added and the reaction heated in a boiling water bath for 7.5 min. The solution was then cooled in an ice-water bath and shaken with 5 ml acid butanol (butan-1-ol plus 5% (v/v) 12 *N* HCl). The absorbance of the butanol layer at 549nm was the read. Pure sialic acid was analyzed as a positive control.

3. RESULTS

3.1 *Burkholderia pseudomallei* Invasion and Intracellular Survival

Burkholderia pseudomallei 1026b is a blood isolate from a lethal case of septicemic melioidosis. *Salmonella typhimurium* 14028s was used as a virulent, invasive control, approximately 5% of the inoculum were able to invade the eukaryotic monolayer. *E. coli* HB101 as a non-invasive control, less than 0.001% on the inoculum were able to invade the eukaryotic monolayer. In all assays, kanamycin (225 μg/ml) was used to kill extracellular bacteria. The MIC for *B. pseudomallei* to kanamycin is 32 μg/ml (data not shown).

3.1.1 Effect of MOI on *B. pseudomallei* Invasion of Type II Pneumocytes

The effect of the multiplicity of infection on *B. pseudomallei* invasion of A549 type II pneumocytes was investigated in order to identify the bacterial load necessary for invasion and to ascertain an appropriate MOI for further assays. MOIs of one, ten or 100 bacteria per A549 cell were examined. Resultant invasion levels were between 0.2% and 1% in all cases (Figure 1). For all further assays, a standard MOI of ten bacteria per eukaryotic cell was used to infect the eukaryotic monolayers.

3.1.2 Comparison of the Invasiveness of *B. pseudomallei* and *B. thailandensis*

B. thailandensis E264, an environmental isolate from Thailand, was investigated to determine its level of invasion in A549 cells compared to the more virulent *B. pseudomallei* 1026b. For *B. pseudomallei*, 1.83% of the initial bacterial load invaded the A549 monolayer as compared to 0.04% of the *B. thailandensis* inoculum (Figure 2). The avirulent *B. thailandensis* strain invaded the A549 monolayer at a signficantly lower rate than the virulent *B. pseudomallei* strain ($p<0.001$).

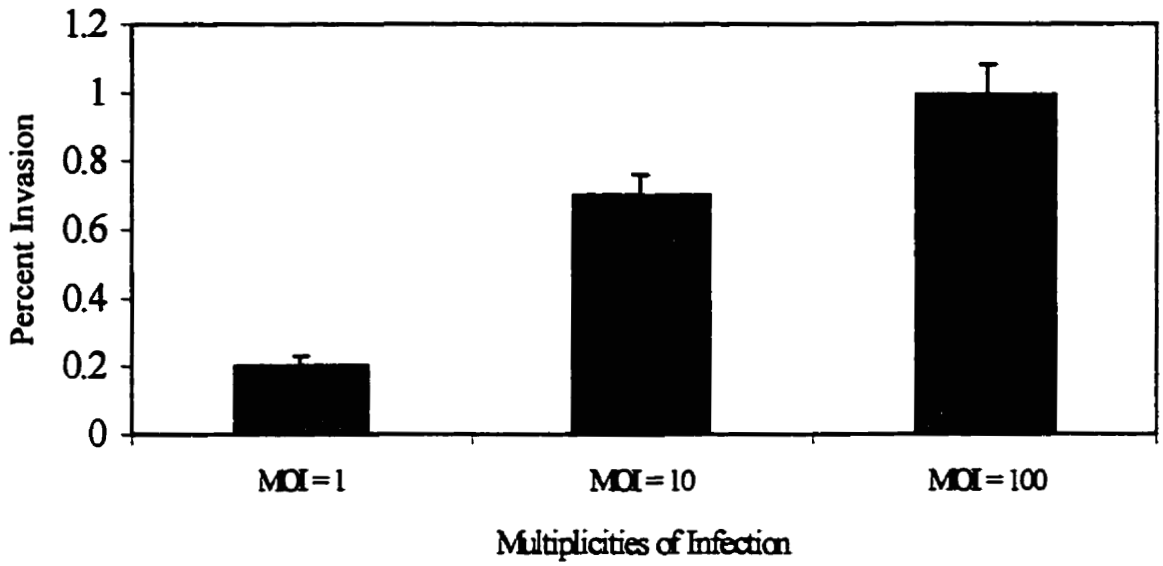


Figure 1. Invasion of A549 type II pneumocytes by *Burkholderia pseudomallei* 1026b at different MOIs. 5×10^5 A549 cells were infected with 25 µl of *B. pseudomallei* diluted to result in multiplicities of infection equal to one, ten or one hundred bacteria per tissue culture cell. Invasion assays were performed as described previously. Data are expressed as the mean of three wells.

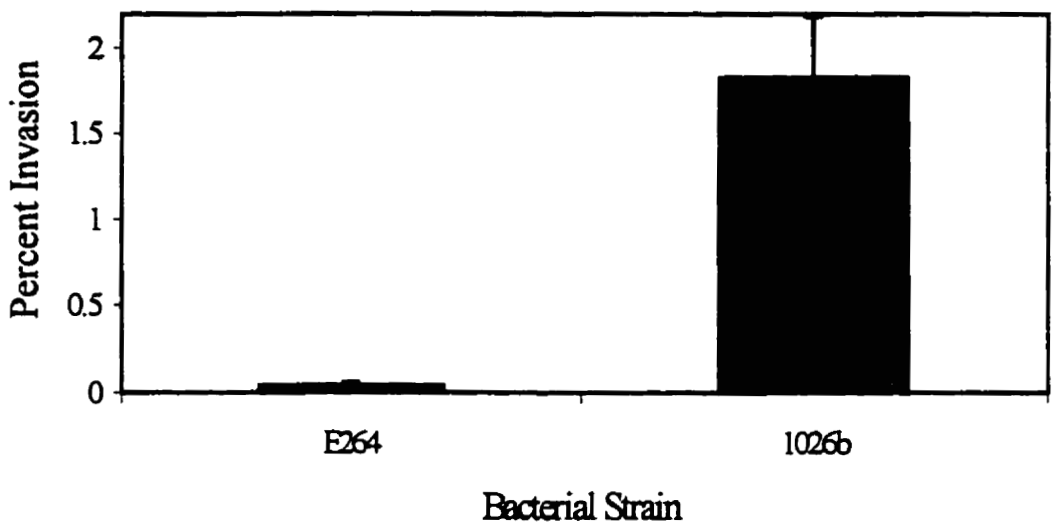


Figure 2. Invasive capabilities of *Burkholderia thailandensis* E264 and *Burkholderia pseudomallei* 1026b in A549 type II pneumocytes. Parallel A549 monolayers were infected by *B. pseudomallei* and *B. thailandensis* at a MOI equal to 10. Invasion assays were performed as previously described. Data are expressed as the mean ± standard deviation of three experiments of three wells each.

3.1.3 Effect of Nocodazole on *B. pseudomallei* Invasion

The rearrangement of host cytoskeletal components is a common element of bacterial invasion. The phenomenon of host tubulin exploitation was examined using nocodazole, a specific inhibitor of microtubule rearrangement. There was no evidence that invasion of A549 type II pneumocytes by *B. pseudomallei* 1026b was inhibited by the presence of nocodazole (Figure 3). A549 monolayers were preincubated with nocodazole dissolved in DMSO or exclusively with DMSO as a control. A basal level of *B. pseudomallei* invasion was established using untreated eukaryotic monolayers. Equivalent amounts of inhibition were seen in the monolayers preincubated with the inhibitor or with the control.

3.1.4 Generation and Characterization of Invasion Deficient Mutants

In order to characterize invasion at the molecular level and identify genetic loci essential for invasion, invasion deficient mutants were generated, using the Tn5-OT182 transposon and screened by qualitative invasion assays.[51] The qualitative invasion assay was exquisitely reliable (Figure 4). In one assay, of six mutants identified, all demonstrated lower rates of invasion as compared to the parental strain. Mutants demonstrating less than ten percent of the parental invasion rates were deemed appropriate for further study. The bacterial genomic DNA flanking the transposon insertion was isolated and sequenced. Homologous sequences were identified using the blastx sequence alignment program to search the sequence databases during July 1999. The nucleotide sequence was translated in all six reading frames and compared to a non-redundant protein sequence database. Twenty-eight transposon-derived mutants were cloned, and sequence homologies were elucidated for eighteen (Table 2). Ten mutants

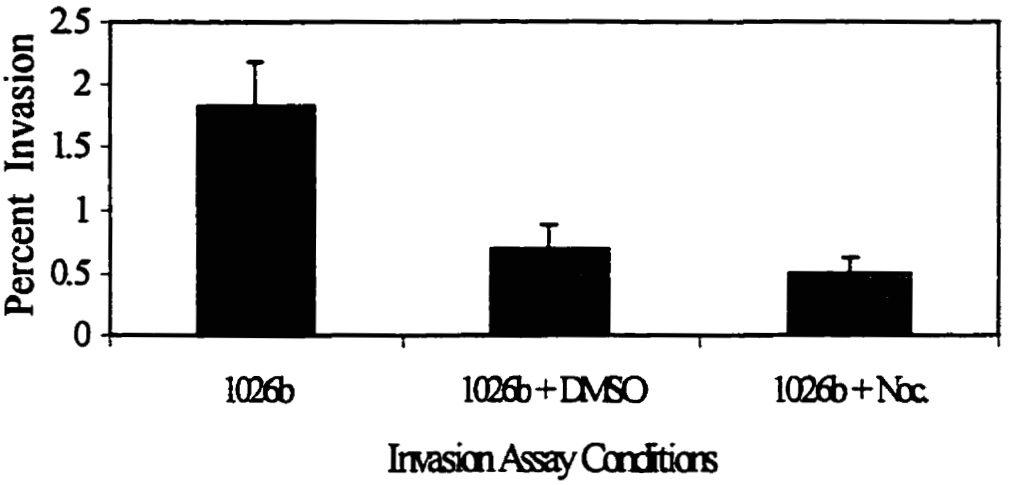


Figure 3. Effect of nocodazole on invasion of A549 type II pneumocytes in *Burkholderia pseudomallei* 1026b. Nocodazole (Noc.) was used to specifically inhibit polymerization of tubulin in order to determine if the exploitation of the host cytoskeletal microtubulin was required for invasion. Eukaryotic monolayers were preincubated with either nocodazole dissolved in dimethylsulfoxide (DMSO) or DMSO alone as a control. A basal level of invasion was established by infecting an untreated monolayer. Invasion assays were performed as previously described. Data are expressed as the mean ± standard deviation of three experiments of three wells each.

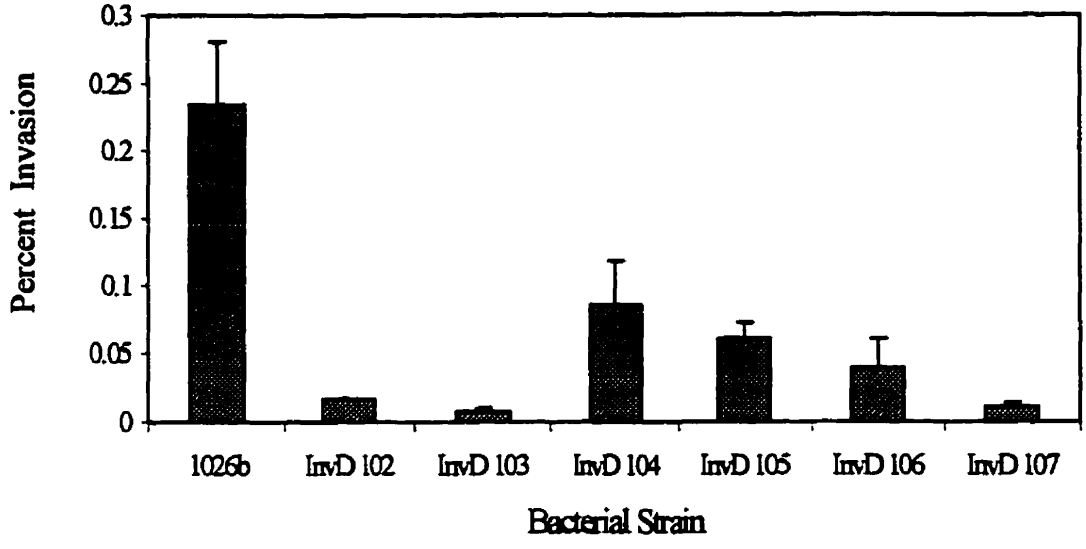


Figure 4. Comparison of the invasion of A549 type II pneumocytes by *Burkholderia pseudomallei* 1026b and its derivative transposon mutants. All invasion deficient mutants were generated by transposon mutagenesis and screened using qualitative invasion assays. The invasion levels of appropriate mutants were quantitated using the previously described invasion assay. Data are expressed as the mean of 3 wells.

demonstrated no significant similarity to any known DNA sequences. These mutants have transposon insertions in genes with functions that have yet to be elucidated. It is probable that these genes are essential either for growth or invasion.

Iron limitation is a condition that intracellular pathogens must overcome to establish a successful infection. Inactivation of iron scavenging genes would therefore lessen the ability of *B. pseudomallei* to invade. Among those invasion-deficient mutants isolated, were mutants containing transposon-inactivated sequences with homology to the DNA sequences of siderophore regulatory proteins (pupRI) in *Pseudomonas putida*; iron binding proteins in *Pasteurella haemolytica, Synechocystis spp., Neisseria gonorrhoeae* or *Neisseria meningitidis,* and iron binding protein precursors in *N. gonorrhoeae, N. meningitidis* or *Ehrlichia chaffeensis.*

There are many types of nutrient limitations that a bacterial cell must overcome to establish a successful endogenous infection. We identified genes that were required for invasion that were also involved in phosphate or carbon starvation responses. The phoB gene product is a response regulator of a two component regulatory system that is induced under phosphate starvation conditions. It induces many genes including those encoding for phosphate starvation inducible proteins of which phoH is a homolog. We also identified a gene involved in invasion that encodes for a protein homologous to the carbon starvation protein A in *E. coli, Helicobacter pylori* and *Mycobacterium tuberculosis.*

We also identified a number of invasion deficient mutants in which the transposn inserted in genes with sequence homology to the DNA sequences that encode for antibiotic resistance proteins. These proteins include bicyclomycin resistance proteins,

Table 2. Genetic loci associated with Tn5-OT182 integrations in *Burkholderia pseudomallei* invasion deficient mutants and the functions of the homologous proteins they encode

Invasion Mutant	Homolog		Function	Level of Homolgy
AJ1D8[51]	CzcRS	*Alcaligenes eutrophus*	Two component regulation of Cd^{2+}, Zn^{2+}, Co^{2+} resistance	
	CopRS	*Pseudomonas syringae*	Two component regulation of Cu^{2+} resistance	
	PcoRS	*Escherichia coli*	Two component regulation of Cu^{2+} resistance	
2DA9	PhoB	*Haemophilus influenzae*	Phosphate regulon transcriptional regulatory protein	4e-05
		Providencia stuartii	Response regulator homolog	7e-05
	AfsQ1	*Streptomyces coelicolor*	Transcriptional regulatory protein	9e-05
	RprY	*Bacteroides fragilis*	Inner membrane signal transducing protein	1e-04
	PhoB	*Pseudomonas aeruginosa*	Phosphate regulon transcriptional regulatory protein	6e-04
		Sinorhizobium meliloti	Phosphate regulatory protein	1e-04
		Lactobacillus sakei	Putative response regulator	2e-04
		Bradyrhizobium japonicum	Phosphate regulatory protein	2e-04
		Mycobacterium leprae	Putative two component response regulator	3e-04
	PhoB	*Shigella dysenteriae*	Phosphate regulon transcriptional regulatory protein	3e-04
	PhoB	*Escherichia coli*	Phosphate regulon transcriptional regulatory protein	3e-04
	GltR	*Pseudomonas aeruginosa*		4e-04
	PhoP	*Yersinia enterocolitica*		6e-04
	PhoB	*Shigella flexneri*	Phosphate regulon transcriptional regulatory protein	6e-04
2AD2	PhoH	*Escherichia coli*	Phosphate starvation inducible protein	1e-07
	PhoH	*Thermotoga maritima*	PhoH related protein	7e-04
2DB2		*Escherichia coli*	ORF 12	2e-38
		Escherichia coli	Hypothetical 77.9KD prt in MRR-TSR intergenic region	6e-38
		Escherichia coli	Carbon starvation gene product	8e-38
	CstA	*Escherichia coli*	Carbon starvation protein A	8e-38
	CstA	*Helicobacter pylori*	Carbon starvation protein A	3e-30
	CstA	*Mycobacterium tuberculosis*	Carbon starvation protein A	4e-25
		Bacillus subtilis	Carbon starvation induction protein	2e-20
		Aquifex aeolicus	Carbon starvation protein A	2e-11

Table 2, continued

2CF3		*Pasteurella haemolytica*	Iron binding protein FbpA precursor	3e-19
		Synechocystis spp.	Periplasmic iron-binding protein	4e-13
		Synechocystis spp.	Iron transport protein	1e-13
	IdiA	*Synechococcus spp.*	Iron starvation protein	4e-12
		Ehrlichia chaffeensis	Iron binding protein precursor	1e-11
		Neisseria gonorrhoeae	Ferric iron-binding protein	2e-05
	fbpA	*Neisseria gonorrhoeae*	Periplasmic iron-binding protein	2e-05
		Neisseria meningitidis	Periplasmic iron-binding protein	2e-05
		Neisseria meningitidis	Major ferric iron binding protein precursor	2e-05
		Neisseria gonorrhoeae	Major ferric iron binding protein precursor	2e-05
2DB3	PupI	*Pseudomonas putida*	Positive siderophore regulatory protein	2e-10
	PupR	*Pseudomonas putida*	Siderophore regulatory protein	1.3e-06
	FecI	*Escherichia coli*	Probable RNA polymerase sigma factor (transcriptional activator)	6e-07
2AF4	PolI	*Haemophilus influenzae*	DNA polymerase I	2e-36
	PolI	*Escherichia coli*	DNA directed DNA polymerase I	5e-36
	PolI	*Streptococcus pneumoniae*	DNA polymerase I	2e-29
2CD9		*Rhizobium meliloti*	C4-dicarboxylate transport protein	1.3e-06
		Rhizobium meliloti	C4-dicarboxylate carrier protein	1.3e-06
		Rhizobium leguminosarum	C4-decarboxylate transport protein (permease)	7.6e-05
2DC10	FucA	*Haemophilus influenzae*	L-fuculose phosphate aldolase	5e-08
	FucA	*Escherichia coli*	L-fuculose phosphate aldolase	7e-09
		Aquifex *aeolicus*	Fuculose-1-phosphate aldolase	2e-05
2DA3		*Escherichia coli*	Putative multimodular enzyme	3e-11
	Nad	*Escherichia coli*	Putative malate oxidoreductase	3e-11
	Nad	*Haemophilus influenzae*	Putative malate oxidoreductase	2e-09
	Tme	*Sinorhizobium meliloti*	NADP-dependent malic enzyme	2e-06
	Tme	*Rickettsia prowazekii*	Malic enzyme	3e-06
	YtsJ	*Bacillus subtilis*	Malate dehydrogenase	7e-06

Table 2, continued

2CE2		*Escherichia coli*	Bicyclomycin resistance (transmembrane) protein	1e-13
	Bcr	*Haemophilus influenzae*	Bicyclomycin resistance protein	4e-10
	Bcr	*Escherichia coli*	Bicyclomycin (sulfonamide) resistance protein	1e-13
		Bacillus subtilis	Bicyclomycin resistance protein	7e-11
	MdfA	*Escherichia coli*	Multidrug resistance (translocase) protein (proton motive force efflux pump)	8e-08
		Salmonella typhimurium	Chloramphenicol and florfenicol resistance protein	4e-10
		Escherichia coli	Drug resistance translocase	4e-10
	Flor	*Salmonella typhimurium*	Putative efflux protein	4e-10
	CmlA	*Pseudomonas aeruginosa*	Chloramphenicol resistance protein	7e-09
	EmrD	*Escherichia coli*	Multidrug resistance protein D (2-module integral membrane pump)	5e-09
	Cmr	*Escherichia coli*	Chloramphenicol resistance pump	8e-08
	Bcr1	*Rickettsia prowazekii*	Bicyclomycin resistance protein	1e-08
2DF2		*Escherichia coli*	Putative transport protein	6e-23
		Campylobacter jejuni	Multidrug efflux transporter	3e-16
		Helicobacter pylori	Multidrug efflux transporter	1e-14
		Bacillus subtilis	Multidrug resistance protein	5e-05
		Brevibacterium linens	Putative efflux protein	1e-04
		Bacillus subtilis	Multidrug resistance efflux transporter 2	8e-04
2CE3		*Streptomyces pristinaespiralis*	Pristinmaycin I synthase 2	3e-20
		Streptomyces virginiae	Virginiamycin S synthetase	5e-20
		Myxococcus xanthus	Saframycin Mx1 synthetase A	6e-20
		Pseudomonas syringae	Syringomycin synthetase	1e-19
	BacA	*Bacillus licheniformis*	Bacitracin synthetase 1	2e-18
		Brevibacillus brevis	Tyrocidine synthetase	6e-18
		Bacillus subtilis	Probable serine activating enzyme	1e-17
	PbsC	*Pseudomonas spp.*	Siderophore biosynthesis protein C	3e-16
	PvdD	*Pseudomonas aeruginosa*	Pyoverdine synthetase D	9e-15

Table 2, continued

2AD5		*Pseudomonas syringae*	Cell division/stress response protein	1e-26
	Ftsk	*Coxiella burnetii*	Cell division protein	2e-23
	Ftsk	*Escherichia coli*	Cell division protein	5e-24
	Ftsk	*Haemophilus influenzae*	Cell division protein	3e-20
	FtsK	*Helicobacter pylori*	Cell division protein	2e-17
	FtsK	*Mycobacterium tuberculosis*	Cell division protein	6e-16
2DD3	f246	*Escherichia coli*	Hypothetical ORF f246	1.1e-07
	f456	*Escherichia coli*	Hypothetical ORF f456	1.4e-23
		Homo sapiens	IgG Fc binding protein	9.4e-05
		Escherichia coli	Putative ATP synthase beta subunit	8e-04
2DC3		*Oryctolagus cuniculus*	Collagen alpha 1 (VIII) chain precursor	2e-06
2DD8	Athb-7	*Arabidopsis thaliana*	Homobox-leucine zipper protein	8.1e-05
		Neurospora crassa	Microtubule binding product p150Glued	1.5e-05
2CF7	pvdD	*Pseudomonas aeruginosa*	Pyoverdine synthetase D	3.8e-05
		Bacillus subtilis	Probable serine activating enzyme	1.5e-05
2CC2	pvdD	*Pseudomonas aeruginosa*	Pyoverdine synthetase D	3.8e-05
	dhbf	*Bacillus subtilis*	Probable serine activating enzyme	1.5e-05
2DC2		no homology elucidated		
2DD2		no homology elucidated		
2DB4		no homology elucidated		
2CD2		no homology elucidated		
2DF3		no homology elucidated		
2DA5		no homology elucidated		
2DA4		no homology elucidated		
InvD102		no homology elucidated		
InvD103		no homology elucidated		
InvD107		no homology elucidated		

chloramphenicol resistance proteins, multidrug resistance proteins and multidrug efflux pumps. In one invasion deficient mutant, the transposon interrupted genes with significant homology to genes that encode synthetic antibiotic synthases and synthetases such as pristinmaycin I synthase 2, Virginamycin S synthetase and saframycin Mx1 synthetase A.

3.1.5 Intracellular Multiplication of *B. pseudomallei* in A549 Cells

The ability of *B. pseudomallei* to survive and replicate in A549 type II pneumocytes was investigated (Figure 5). A basal level of intracellular organisms was established at 4 hours post invasion. By six hours post invasion, there was a 1.6-fold increase in the number of intracellular organisms. After an additional six hours, there was a 6.9-fold increase in the number of intracellular bacteria from the basal level established at four hours. By 24 hours post infection, the number of intracellular organisms had increased by 33-fold. The intracellular existence of *B. pseudomallei* was confirmed by electron microscopy. Figure 6 and Figure 7 show transmission electron micrographs of *B. pseudomallei* 1026b at 12 and 16 hours post infection, respectively. Intracellular bacteria are clearly visible in membrane bound vacuoles. Intracellular replication and the presence of multiple bacteria in a single vacuole are also observed.

3.1.6 Evidence of Phagosome-Lysosome Fusion in Host Cells

The occurrence of a phagosome-lysosome fusion in A549 type II pneumocytes infected with *B. pseudomallei* was assessed using confocal fluorescent microscopy. The green fluorescent protein of *Aequorea victoria* was cloned into the pUCP28T plasmid and conjugated into *B. pseudomallei* 1026b. Using confocal microscopy, the pathogen could be identified in tissue culture by its emitted green fluorescence. In the confocal

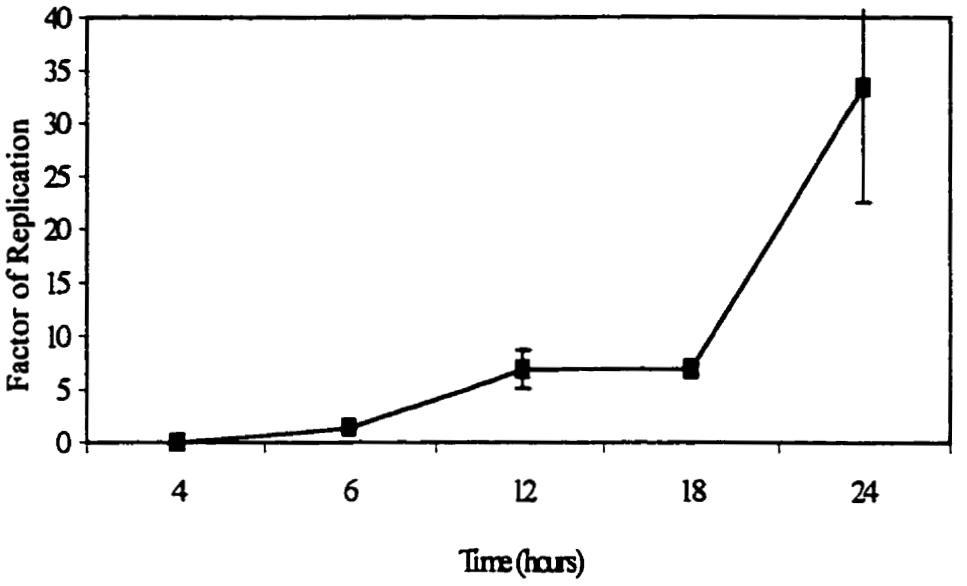


Figure 5. Intracellular multiplication of *Burkholderia pseudomallei* 1026b in A549 type II pneumocytes. The number of intracellular organisms at four hours was determined as a basal level. The number of organisms at six, twelve, eighteen, and twenty-four hours was determined, and the factor of replication calculated. Data are expressed as the mean ± standard deviation of three experiments of three wells each.

Figure 6. Transmission electron micrograph of *Burkholderia pseudomallei* 1026b infected A549 type II pneumocytes, 12 hours post infection. *B. pseudomallei* are contained in membrane bound vacuoles. Magnification x 20235.

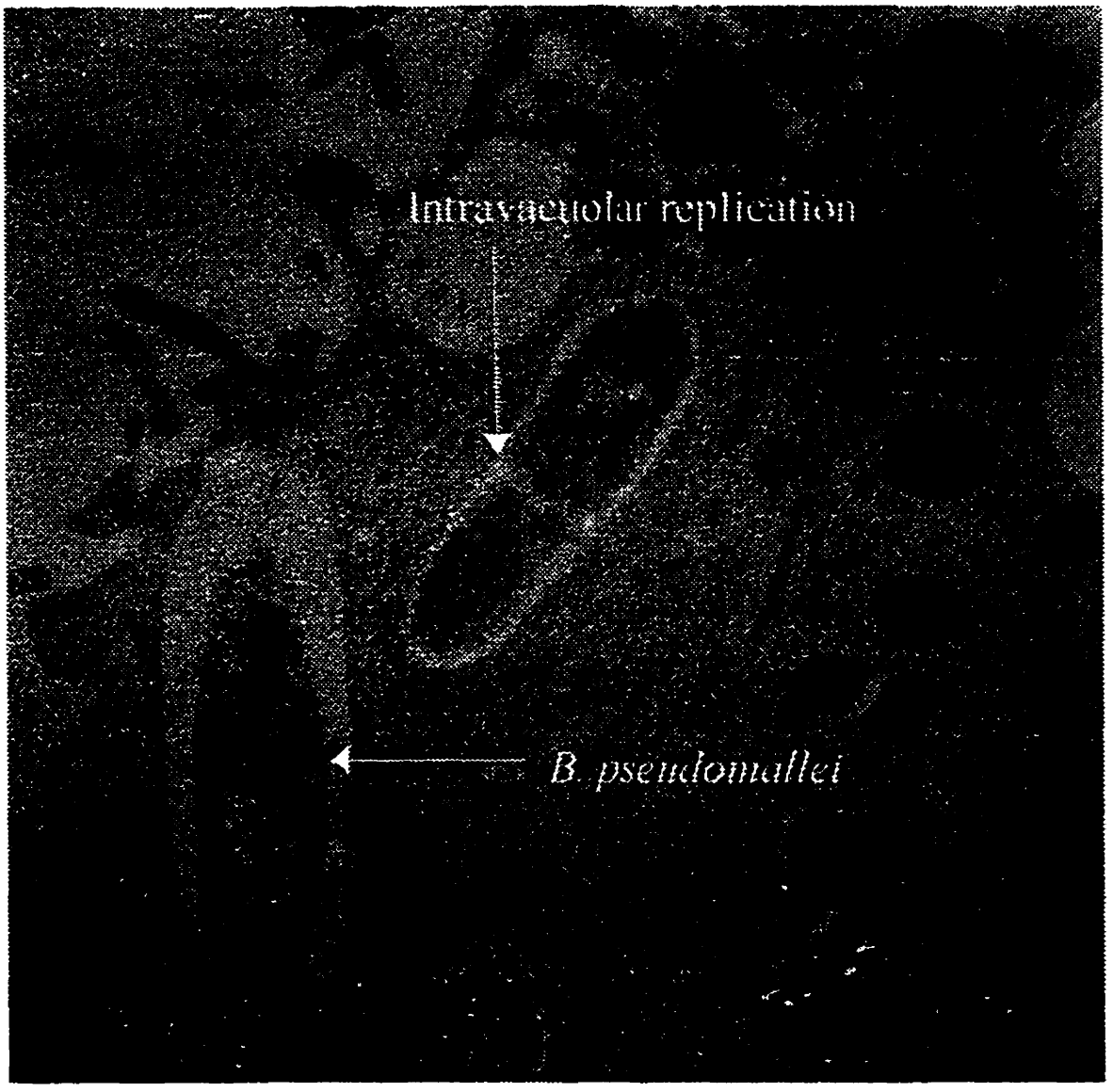


Figure 7. Transmission electron micrograph of *Burkholderia pseudomallei* 1026b infected A549 type II pneumocytes, 16 hours post infection. *B. pseudomallei* are contained in membrane bound vacuoles. The micrograph shows evidence of the intracellular multiplication of *B. pseudomallei* in membrane bound vacuoles. Magnification x 31825.

micrograph, some bacteria did not emit green fluorescence. Pathogens have been shown to lose the GFP protein while in tissue culture. As well, in confocal microscopy, the images are captured in a stack of cross-sections. As one moves through the stack of images, the individual sections (and fluorescence) will go into and then out of focus. The lysosomes were labeled with Lysotracker Red which accumulates in the lysosomes due to the intracellular acidity there. This probe emits a red fluorescence that is somewhat photosensitive. Initially, the level of invasion of the constructed *B. pseudomallei* 1026b (pUCP28Tgfp1) was examined to ensure it was comparable to that of the parental strain, *B. pseudomallei* 1026b (Figure 8). Invasion rates for both strains were found to be within 20 percent of each other. In this assay, 0.81% of the initial inoculum of *B. pseudomallei* 1026b infected the A549 monolayer, while 0.68% of the initial inoculum of *B. pseudomallei* 1026b(pUCP28Tgfp) infected the A549 monolayer. As the invasion rates were comparable, intracellular survival assays were performed as described using *B. pseudomallei* 1026b(pUCP28Tgfp1) as the infecting pathogen. The intracellular bacteria were allowed to replicate overnight in the A549 monolayer, and colocalization of fluorescence was assessed using confocal microscopy (Figure 9). A differential interference contrast filter was used to view cellular structures. A fusion event was determined by the colocalization of red and green fluorescence.

3.2 *Burkholderia pseudomallei* Response to Acidic Conditions

3.2.1 Response of *B. pseudomallei* to Heavy Metal Cations

One invasion deficient mutant, AJ1D8, was characterized and found be sensitive to cadmium.[51] Vacuolar-type ATPases that regulate the intracellular pH in leukocytes

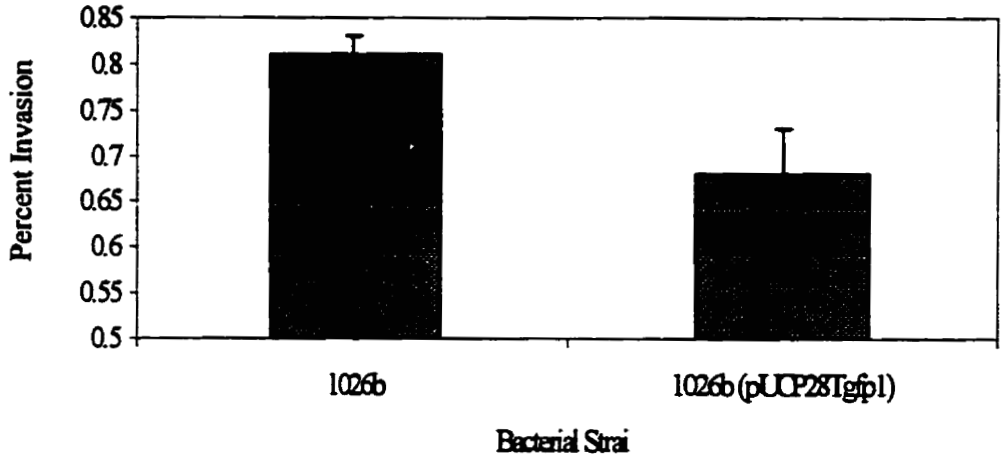


Figure 8. Effect of the insertion of the GFP protein on the invasion of A549 type II pneumocytes by *Burkholderia pseudomallei* 1026b. A549 monolayers were infected in parallel by *B. pseudomallei* 1026b and *B. pseudomallei* 1026b (pUCP28Tgfp1) at a MOI equal to 10. Invasion assays were performed as previously described. Data are expressed as the mean of three wells each.

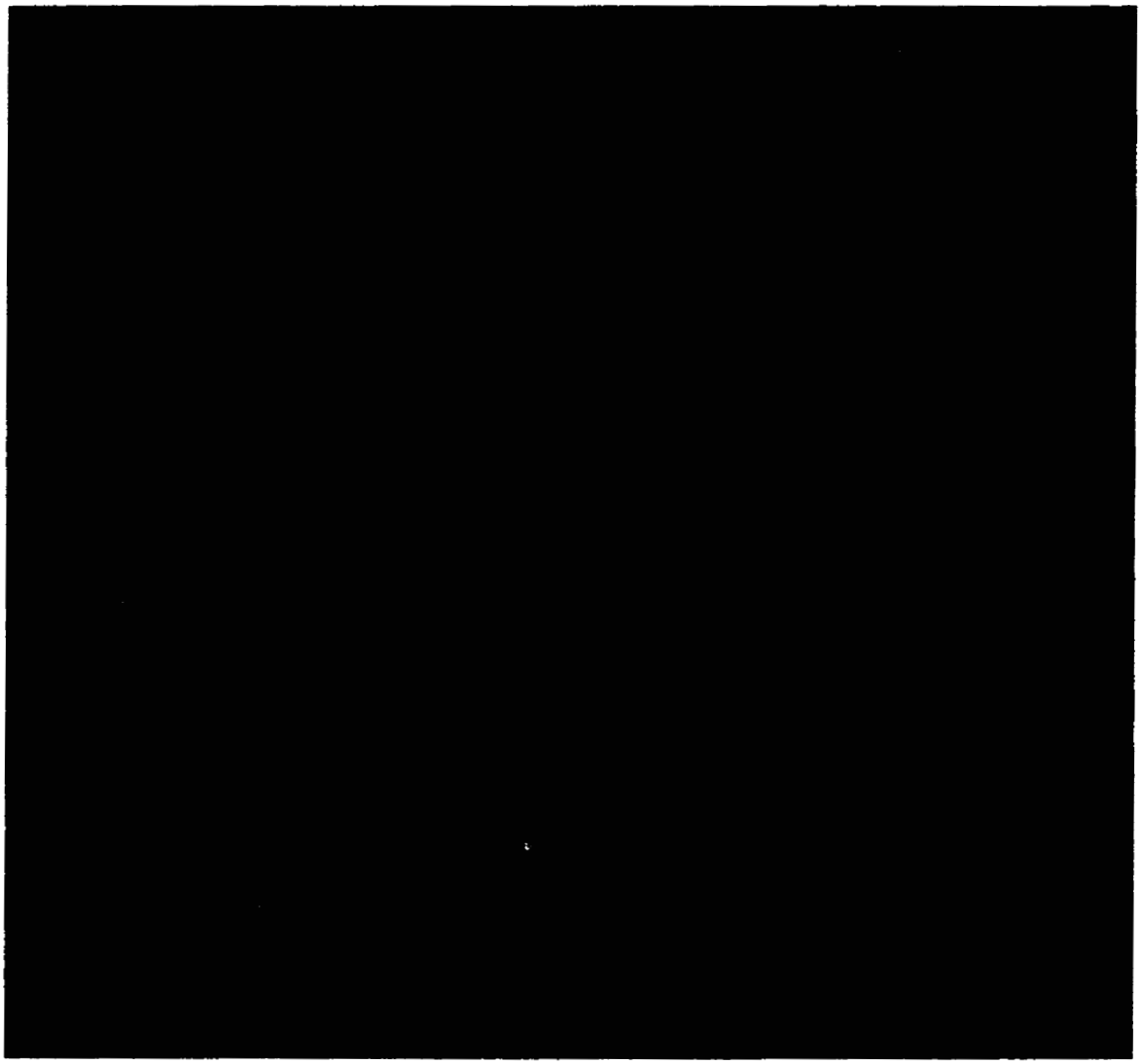


Figure 9. Confocal fluorescent micrograph of *Burkholderia pseudomallei* 1026b (pUCP28Tgfp1) infected A549 type II pneumocytes establishing evidence of a phagosome-lysosome fusion. Lysosomes are labeled with rhodamine. A phagosome-lysosome fusion event was identified by the colocalization of fluorescence. Magnification x 100.

also require cadmium in order for optimal function. As previously shown, *B. pseudomallei* infected vacuoles undergo a fusion event with host lysosomes. This fusion results in the release of a number of acid hydrolases into the bacteria containing vacuoles. The pH of the lysosome is known to be acidic in order for optimal function of these hydrolytic enzymes. *B. pseudomallei* must therefore be able to survive in an acidic environment in order to establish a successful endogenous infection. We speculated that resistance to lysosomal acidification may be due to the presence of a cadmium sensitive ATPase. We established that *B. pseudomallei* 1026b is resistant to 5 mM cadmium while AJ1D8 is sensitive (Table 3). The minimum inhibitory concentrations of several heavy metal cations were established for both *B. pseudomallei* 1026b and AJ1D8 (Table 4). The only differences in the MICs of the mutant and parent strains were seen in the presence of cadmium and zinc. The MIC for cadmium was established as 9.6 mM for *B. pseudomallei* 1026b and 0.6 mM for AJ1D8.[51]

3.2.2 Evaluation of the Intracellular pH of *B. pseudomallei* in Acidic Conditions

Oregon green dye was used to evaluate the intracellular pH of *B. pseudomallei* (Table 5). This dye emits green fluorescence in an acidic environment. After incubation for 15 or 30 minutes at pH 3, pH 5, or pH 7, the bacterial cells were viewed to determine the percentage of cells fluorescing. As expected at pH 7, a neutral pH, there were essentially no bacterial cells with an acidic intracellular environment. However, at pH 5, four to five percent of all *B. pseudomallei* demonstrated fluorescence, indicating that the mechanisms which mediate the efflux of protons from the bacterial cytoplasm had been overwhelmed. It was expected that this number would increase as the pH in the

Table 3. Sensitivity of *Burkholderia pseudomallei* 1026b and its derivative transposon mutant AJ1D8 to cadmium.

Strain	Cadmium Sensitivity [a]
1026b	Resistant
AJ1D8	Sensitive

Overnight bacterial cultures were adjusted to McFarland's standard 0.5 and plated on TSA supplemented with cadmium.

[a] Resistance to cadmium is defined as growth on TSA plates supplemented with 5mM Cd^{2+} after 48 hours incubation at 37°C.

Table 4. Minimum inhibitory concentrations of heavy metal cations for *Burkholderia pseudomallei* 1026b and the invasion mutant AJ1D8.

Metal Cation	MIC (mMol/l) [a]	
	1026b	AJ1D8
Copper	9.6	9.6
Cadmium	9.6	0.6
Cobalt	1.2	1.2
Nickel	4.8	4.8
Magnesium	>38.4	>38.4
Zinc	4.8	2.4

MICs were performed in concentrations of cations diluted in Mueller Hinton Broth and inoculated with overnight bacterial cultures adjusted to McFarland's Standards 0.5 and then diluted 1:10.

[a] MIC is defined as the lowest concentration of the metal cation able to inhibit growth after incubation for 24 hours at 37°C.

Table 5. Evaluation of *Burkholderia pseudomallei* 1026b intracellular pH after incubation in sodium citrate buffers.

Media pH	Time (min.)[b]	% of *B. pseudomallei* 1026b with Low Intracellular pH [a]
7	15	0.50
	30	0.32
5	15	5.31
	30	4.06
3	15	0
	30	0

[a] Intracellular pH was evaluated using Oregon Green Dye dissolved in dimethylformamide. At low pH, the dye emits a green fluorescence using the bacterial cell's metabolic processes. All percentages are the mean of 10 fields of view.

[b] *B. pseudomallei* 1026b was incubated in the sodium citrate media with 10 μM Oregon Green Dye and evaluated immediately for fluorescence.

Table 6. Survival of *Burkholderia pseudomallei* 1026b after challenge at low pH

Media pH	Time of Challenge (min.)	Survival of *B. pseudomallei* 1026b [a]
7	15	+++
	30	+++
5	15	++
	30	++
3	15	++
	30	++

[a] Survival was assessed qualitatively by growth on LB plates after 48 hrs incubation at 37°C. Overnight cultures of *B. pseudomallei* grown in LB broth were plated and used to represent +++ survival.

environment decreased. However, at pH 3, there were no bacterial cells emitting any fluorescence. It was hypothesized that *B. pseudomallei* was killed at such a low pH. Oregon green dye cannot function without the active metabolic processes of the bacterial cell. Thus, there would be no fluorescence if the bacteria were not viable.

3.2.3 Survival of *B. pseudomallei* after Acidic Challenge

Survival of *B. pseudomallei* was qualitatively assessed after 15 and 30 minute challenges at pH 3, pH 5, and pH 7 (Table 6). By visual comparison, this pathogen was shown to successfully survive challenges at all pHs. The effect of a 30 minute challenge at pH 3 was then assessed by quantitating the number of bacteria that survived (Figure 10). A control assay in which the bacteria were not subjected to an acidic challenge was tested in parallel and used to represent 100 percent survival. Approximately 69% of *B. pseudomallei* 1026b survived the 30 minutes challenge at pH 3. The addition of excess cadmium to the challenge environment resulted in increased (approximated 96%) survival. However, excess zinc appeared to hinder the *B. pseudomallei* response to a pH 3 challenge and resulted in only 35% survival.

3.2.4 Evaluation of Optimal pH for *B. pseudomallei* Growth

Further studies into the physiological response of *B. pseudomallei* to an acid environment were initiated. In order to determine the optimal pH for this pathogen's growth, *B. pseudomallei* was subcultured 1:250 into a graded pH series of sodium citrate buffers. After 24 hours at 37°C, 250 RPM, the optical density (600 nm) was determined (Table 7). At pH 4.4 the optical density was 1.1136; however there was a dramatic

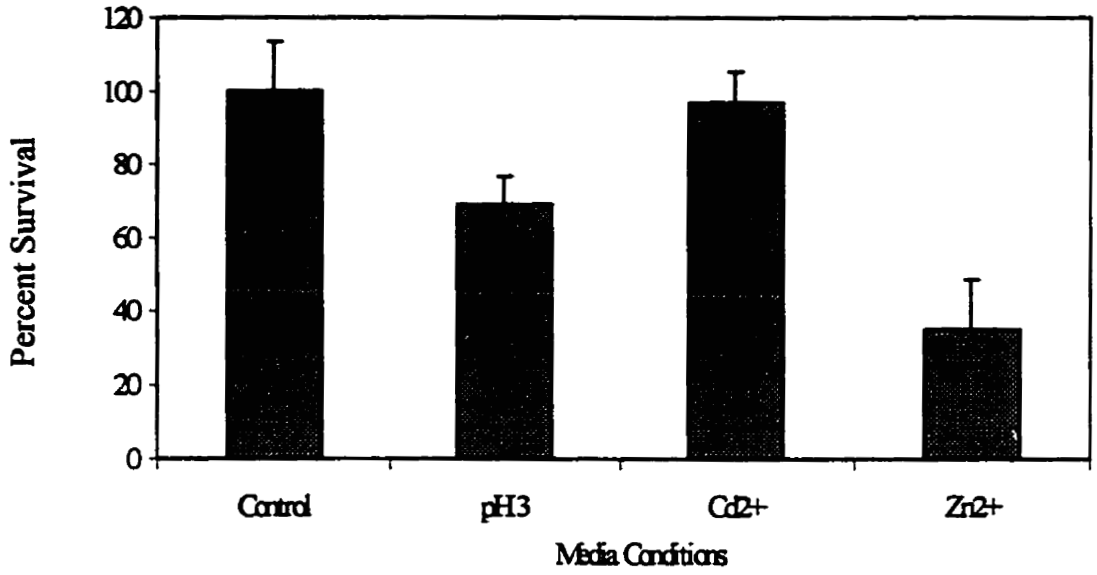


Figure 10. Effect of acidic challenge on *Burkholderia pseudomallei* 1026b survival. After a 30 minutes challenge in a pH 3 sodium citrate/HCl buffer, the number of bacteria that survived were quantitated by plating serial dilutions. A control sample that had not been challenged was tested in parallel and used to represent 100 percent survival. Samples were also challenged in pH 3 sodium citrate buffer supplemented with cadmium (Cd^{2+}) or zinc (Zn^{2+}) to investigate whether either cation could ameliorate the survival of *B. pseudomallei* under acidic condtions

Table 7. Evaluation of optimal pH for growth of *Burkholderia pseudomallei* 1026b in sodium citrate buffer.

Media pH	OD 600nm
5.0	1.15
4.5	1.05
4.4	1.11
4.3	0.16
4.2	0.11
4.1	0.08
4.0	0.08
3.5	0.03
3.0	0.03

Overnight cultures of *B. pseudomallei* 1026b grown in LB were subcultured 1:250 to sodium citrate buffers supplemented with glucose and NH_4CL and incubated for 24 hours at 37°C, 250 RPM. Growth was assessed by absorbance at 600nm.

decrease in optical density to 0.1593 at pH 4.3. The lowest pH which allowed for optimal growth was determined to be pH 4.5 and was used for all further experimentation.

3.2.5 Urease Production by *B. pseudomallei* in Acidic Conditions

Helicobacter pylori survives the acid pH of the stomach by secreting urease which converts urea to ammonia and carbon dioxide. The bacterium surrounds itself with this ammonia which neutralizes the acid in the immediate area. As *B. pseudomallei* must also survive in an acid environment, production of urease by *B. pseudomallei* was investigated. There was no evidence of urease production when *B. pseudomallei* was grown in pH 4.5 sodium citrate buffer or in LB which has an approximate pH of 7 (Table 8). In order to investigate the presence of an inducible urease, M9 media containing urea as a sole nitrogen source was inoculated with *B. pseudomallei.* Bacterial growth will only occur if a bacterial urease is present to cleave the urea and release free nitrogen for bacterial utilization. After 24 hours (37°C, 250 RPM), there was abundant bacterial growth as evidenced by the visible turbidity. However, analysis of both the whole cell suspension and the supernatant using Bacto urea broth revealed no evident urease production (Table 9).

3.2.6 Effect of pH on Optical Density and Growth of *B. pseudomallei*

When long term survival of *B. pseudomallei* in pH 4.5 buffer was assessed at 0 hours and 48 hours by measuring optical density (600nm) and bacterial numbers (cfu/ml), an interesting observation was noted. A bacterial culture was subcultured in parallel into

Table 8. Production of urease by *Burkholderia pseudomallei* 1026b grown *in vitro* in LB broth or pH4.5 sodium citrate/HCl buffer.

Growth Conditions	Production of Urease[a]
Positive Control (NaOH)	+++
Negative Control (sd H_2O)	-
1026b Whole cell in LB	-
1026b Supernatant in LB	-
1026b Whole cell in pH4.5 buffer	-
1026b Supernatant in pH4.5 buffer	-

B. pseudomallei was grown in LB broth and pH 4.5 sodium citrate/HCl buffer supplemented with glucose and ammonium chloride. The whole cell and supernatant fractions were assayed for the production of urease.

[a] Urease production is identified by cerise colour in Bacto urea broth after stationary incubation for 24 hours at 37°C.

Table 9. Production of inducible urease by *Burkholderia pseudomallei* 1026b grown in M9 media using urea as a sole nitrogen source.

Growth Conditions	Production of Urease[a]
Positive Control (NaOH)	+++
Negative Control (sd H_20)	-
1026b Whole cell in LB	-
1026b Supernatant in LB	-
1026b Whole cell in M9 media + Urea	-
1026b Supernatant in M9 media + Urea	-

B. pseudomallei was grown in LB broth and M9 media supplemented with urea. The whole cell and supernatant fractions were assayed for the production of urease.

[a] Urease production is identified by cerise colour in Bacto urea broth after stationary incubation for 24 hours at 37°C.

LB broth and pH 4.5 buffer resulting in equivalent cell numbers and optical density. After 48 hours (37°C, 250 RPM), there was an increase in optical density in the pH 4.5 culture that could not be accounted for by an increase in bacterial cell numbers (Figure 11 and Figure 12).

3.2.7 Growth of *B. pseudomallei* at Normal and Acidic pHs.

The growth of *B. pseudomallei* 1026b in LB and in pH 4.5 sodium citrate buffer supplemented with glucose and ammonium chloride was examined. When *B. pseudomallei* was grown in LB, at pH 7, there was a logarithmic increase in both optical density and cell number as expected (Figure 13). In the sample grown in pH 4.5 buffer, there was the same trend as previously observed; over 48 hours, there was an increase in optical density that was not correlated with a corresponding increase in cell number (Figure 14). It was also observed that *B. pseudomallei* grew at a slower rate in the pH 4.5 buffer.

3.3 Identification and Characterization of the Exopolysaccharide Produced by *Burkholderia pseudomallei* at Acidic pH

3.3.1 Exopolysaccharide Production by *B. pseudomallei* at Acidic pH

The hypothesis that the increase in optical density observed when *B. pseudomallei* is incubated at an acidic pH is due to the secretion of an exopolysaccharide was confirmed using the orcinol-sulphuric acid assay for reducing sugars. After 24 hours growth in pH 4.5 buffer, the orcinol assay indicated an increase in the amount of polysaccharide in both the whole cell and supernatant fractions (Figure 15). There was

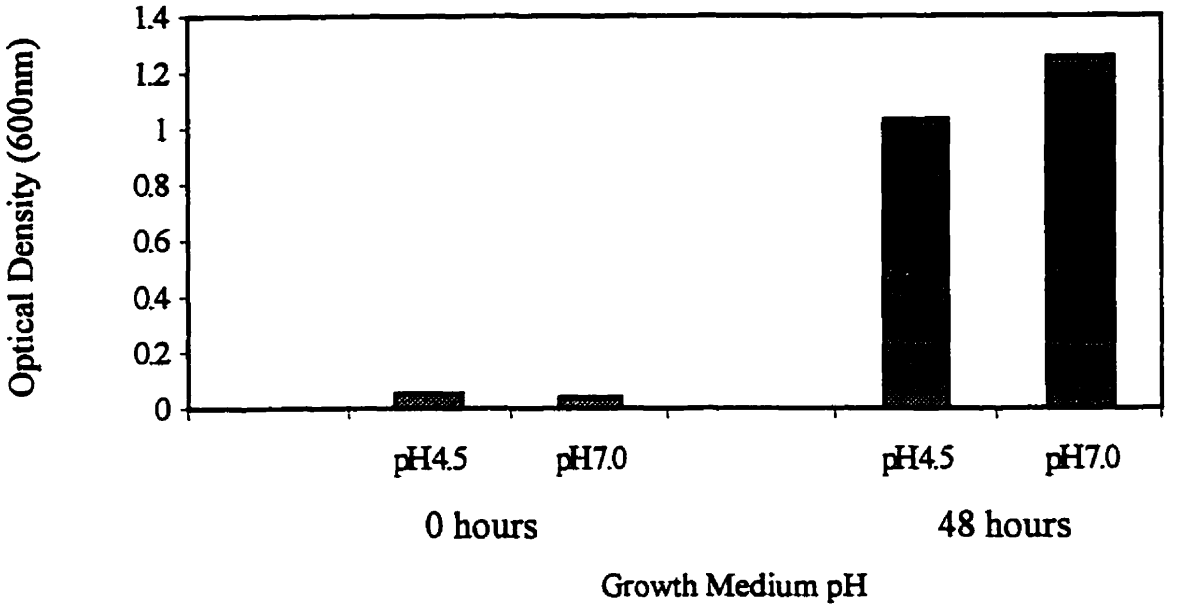


Figure 11. Effect of pH on optical density of *Burkholderia pseudomallei* 1026b *in vitro*. *B. pseudomallei* 1026b was grown overnight in LB and subcultured in parallel into LB broth and pH 4.5 sodium citrate buffer. After 48 hours (37°C, 250 RPM), the optical density (600 nm) was assessed.

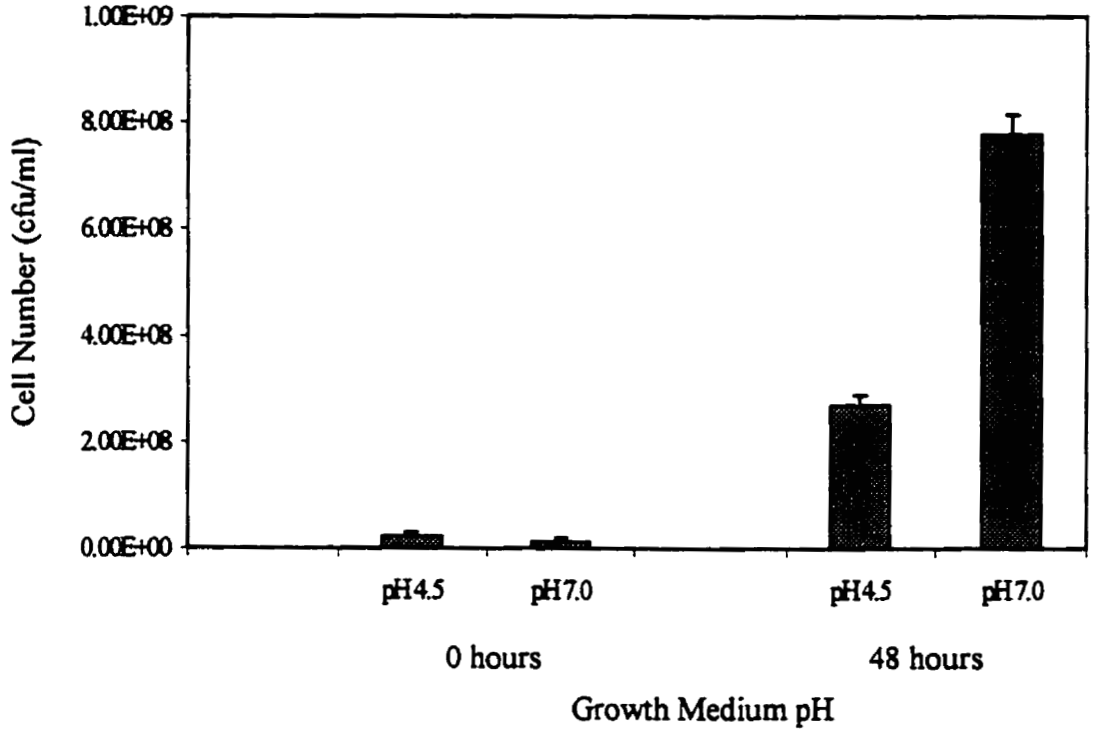


Figure 12. Effect of pH on cell number of *Burkholderia pseudomallei* 1026b *in vitro*. *B. pseudomallei* 1026b was grown overnight in LB and subcultured in parallel into LB broth and pH 4.5 sodium citrate buffer. After 48 hours (37°C, 250 RPM), the cell number (cfu/ml) was assessed.

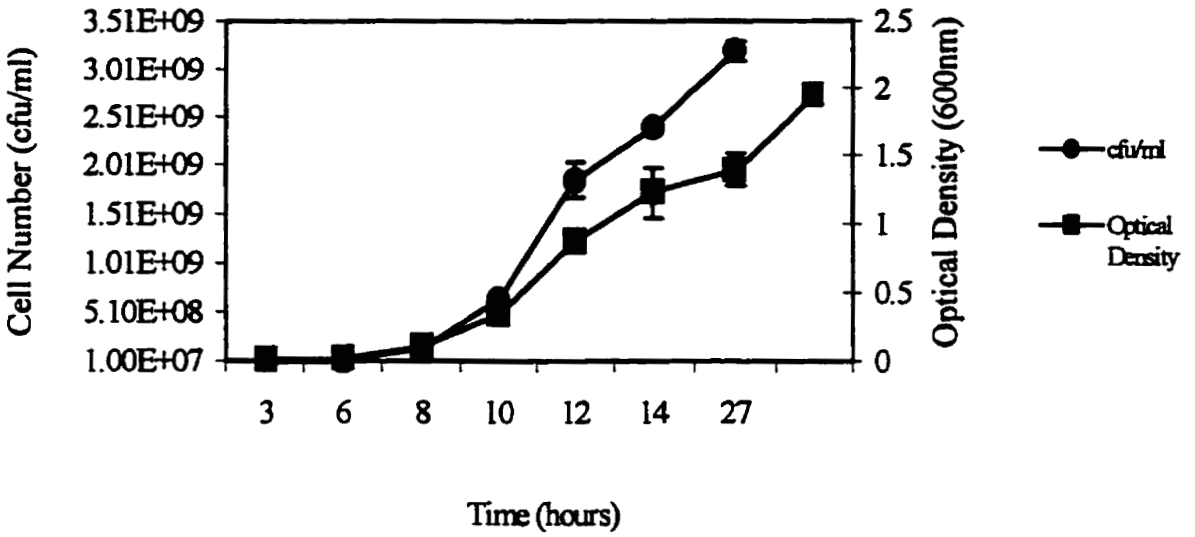


Figure 13. Growth of *Burkholderia pseudomallei* 1026b in LB broth. *B. pseudomallei* 1026b was grown overnight in LB and subcultured into LB broth. At appropriate time points, growth was assessed by cell number (cfu/ml) and optical density (600 nm).

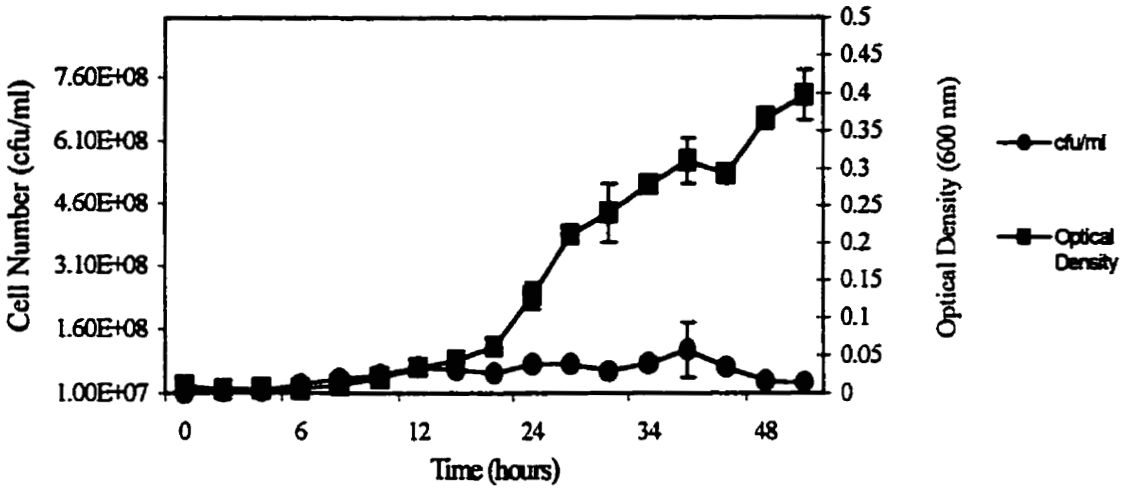


Figure 14. Growth of *B. pseudomallei* 1026b in pH 4.5 buffer. *B. pseudomallei* 1026b was grown overnight in LB and subcultured into pH 4.5 sodium citrate buffer. At appropriate time points, growth was assessed by cell number (cfu/ml) and optical density (600 nm).

eight times more EPS produced at pH 4.5 than in LB in the whole cell fraction and nine times more EPS produced at pH 4.5 than in LB in the supernatant. There was also an increase in EPS production after 48 hours at pH 4.5 (Figure 16). There was five times more EPS in the whole cell fraction and four times more EPS in the supernatant from the pH 4.5 samples compared to the LB samples.

3.3.2 Initial Characterization of Crude EPS

Initial characterization of the crude EPS was performed using *B. pseudomallei* grown overnight and subcultured in parallel into pH 4.5 sodium citrate/HCl buffer and LB buffer. Whole cell bacterial suspensions in pH 4.5 buffer and LB were assayed for the presence of capsule, LPS Type I and LPS Type II to assess any increase in these cell surface components under acidic conditions.

A capsular polysaccharide composed of three galactoside-KDO repeats with O-acetylation at the 2-position has been identified in *B. pseudomallei.* [44,45] Using a monoclonal antibody to this capsule (mAb IgG 3015), the presence of this capsule was investigated by ELISA (Table 10). The mAb IgG 3015 reacted less with the *B. pseudomallei* culture grown in pH 4.5 buffer than with the *B. pseudomallei* grown in LB. Thus the increase in optical density at low pH could not be accounted for by an increase in capsule production.

The possibility that the increase in optical density was due to increased LPS production was also investigated (Table 11). *B. pseudomallei* produces two types of LPS. LPS Type I has an O-antigen composed of 2-O acetyl-6-deoxy manno-heptose repeats.

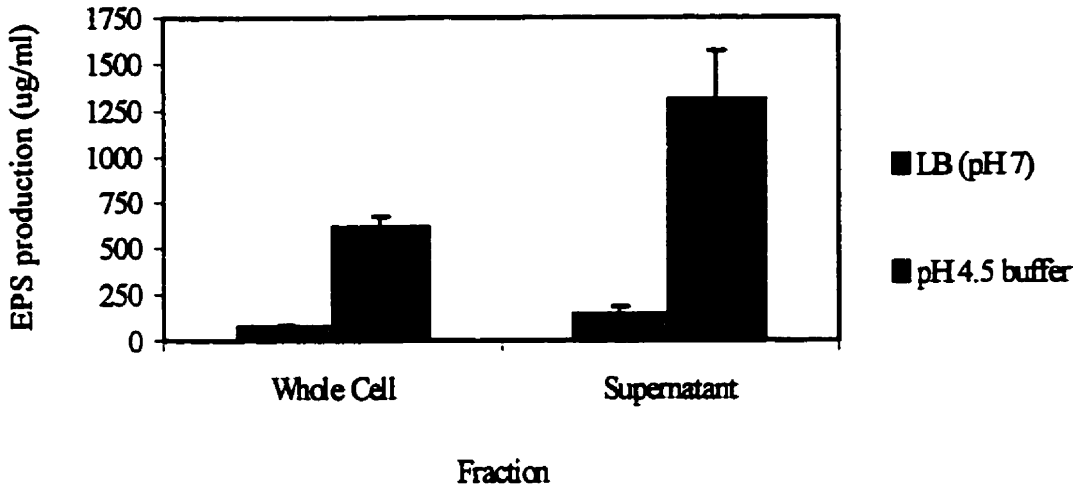


Figure 15. Production of extracellular polysaccharide *in vitro* by *Burkholderia pseudomallei* 1026b after 24 hours at different pHs. *B. pseudomallei* was grown overnight in LB broth and subcultured into pH 4.5 sodium citrate buffer. At 24 hours (37°C, 250 RPM), the whole cell fraction and the supernatant were tested for the amount of polysaccharide produced using the orcinol-sulphuric acid assay. A dextran standard curve was assayed in parallel to allow for quantification. The supernatant was obtained by centrifugation of the whole cell fraction and removal of the top layer by pipetting.

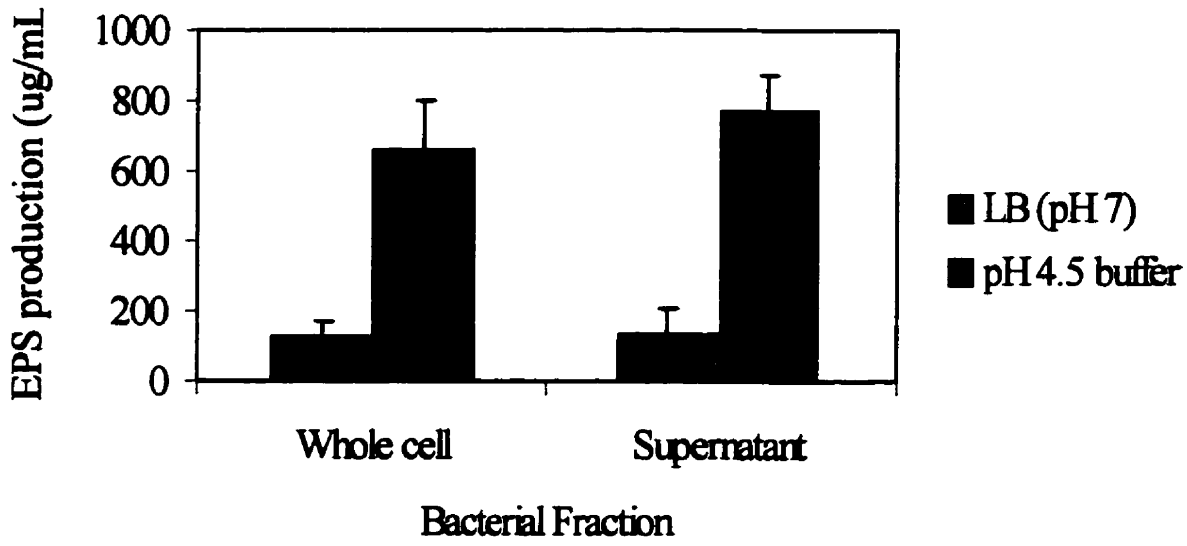


Figure 16. Production of extracellular polysaccharide *in vitro* by *Burkholderia pseudomallei* 1026b after 48 hours at different pHs. *B. pseudomallei* was grown overnight in LB broth and subcultured into pH 4.5 sodium citrate buffer. At 48 hours (37°C, 250 RPM), the whole cell fraction and the supernatant were tested for the amount of polysaccharide produced using the orcinol-sulphuric acid assay. A dextran standard curve was assayed in parallel to allow for quantification. The supernatant was obtained by centrifugation of the whole cell fraction and removal of the top layer by pipetting.

The O-antigen of LPS Type II is a D-glucose β-1,3-6 deoxy-L-talose repeat. Using a polyclonal antibody to the LPS Type I O-antigen and a monoclonal antibody to the LPS Type II O-antigen, the presence of LPS was investigated by ELISA. There was a slight increase in the reaction of the polyclonal antibody to LPS Type I O-antigen in the sample that was grown in pH 4.5 buffer. There was also an increase in the reaction of the monoclonal antibody to LPS Type II O-antigen in the sample that was grown in pH 4.5 buffer. It appears that the increase in optical density that occurs when *B. pseudomallei* is grown in pH 4.5 buffer may be partially accounted for by an increase in LPS, specifically in the Type II LPS.

3.3.3 Initial Characterization of Pure EPS Fractions

The exopolysaccharide was isolated as described (Figure 17). The isolation procedure resulted in two sets of fractions, one set was isolated from the ethanol precipitate (called EP fractions), and the other set was isolated from residue after evaporating off the ethanol supernatant (called RVR fractions). The fractions isolated from the size exclusion column were assayed for the presence of a polysaccharide using the orcinol-sulphuric acid assay. The fractions were then assayed for the presence of any residual glucose from the growth media, as well as for the presence of KDO and sialic acid.

The pooled column fractions were assayed for the presence of carbohydrate (Table 12). As a positive control, dextran sulphate (2 mg/ml) was assayed concurrently with the pooled column fractions. All fractions assayed demonstrated an orcinol reaction

Table 10. Demonstration of the presence of capsule in *Burkholderia pseudomallei* 1026b grown *in vitro* at different pHs.

	B. pseudomallei 1026b Culture Media	
	LB	pH 4.5 buffer
Reaction with Capsule mAb [a]	0.18	0.14

Overnight cultures of *B. pseudomallei* 1026b were subcultured in parallel into LB broth and pH 4.5 sodium citrate buffer. After 24 hours (37°C, 250 RPM), the presence of capsule was assayed.

[a] Elisa results are presented as comparisons of optical density at 405nm using monoclonal antibody IgG 3015 to a *B. pseudomallei* capsule.

Table 11. Demonstration of the presence of Type I and Type II LPS in *Burkholderia pseudomallei* 1026b grown *in vitro* at different pHs.

Reaction of	*B. pseudomallei* 1026b Culture Media	
O-antigen [a]	LB	pH 4.5 buffer
Type I LPS	0.33	0.45
Type II LPS	0.29	0.58

Overnight cultures of *B. pseudomallei* 1026b were subcultured in parallel into LB broth and pH 4.5 sodium citrate buffer. After 24 hours (37°C, 250 RPM), the presence of LPS Type I and LPS Type II were assayed.

[a] Elisa results are presented as comparisons of optical density at 405nm using antibody to the Type I O-antigen and Type II O-antigen.

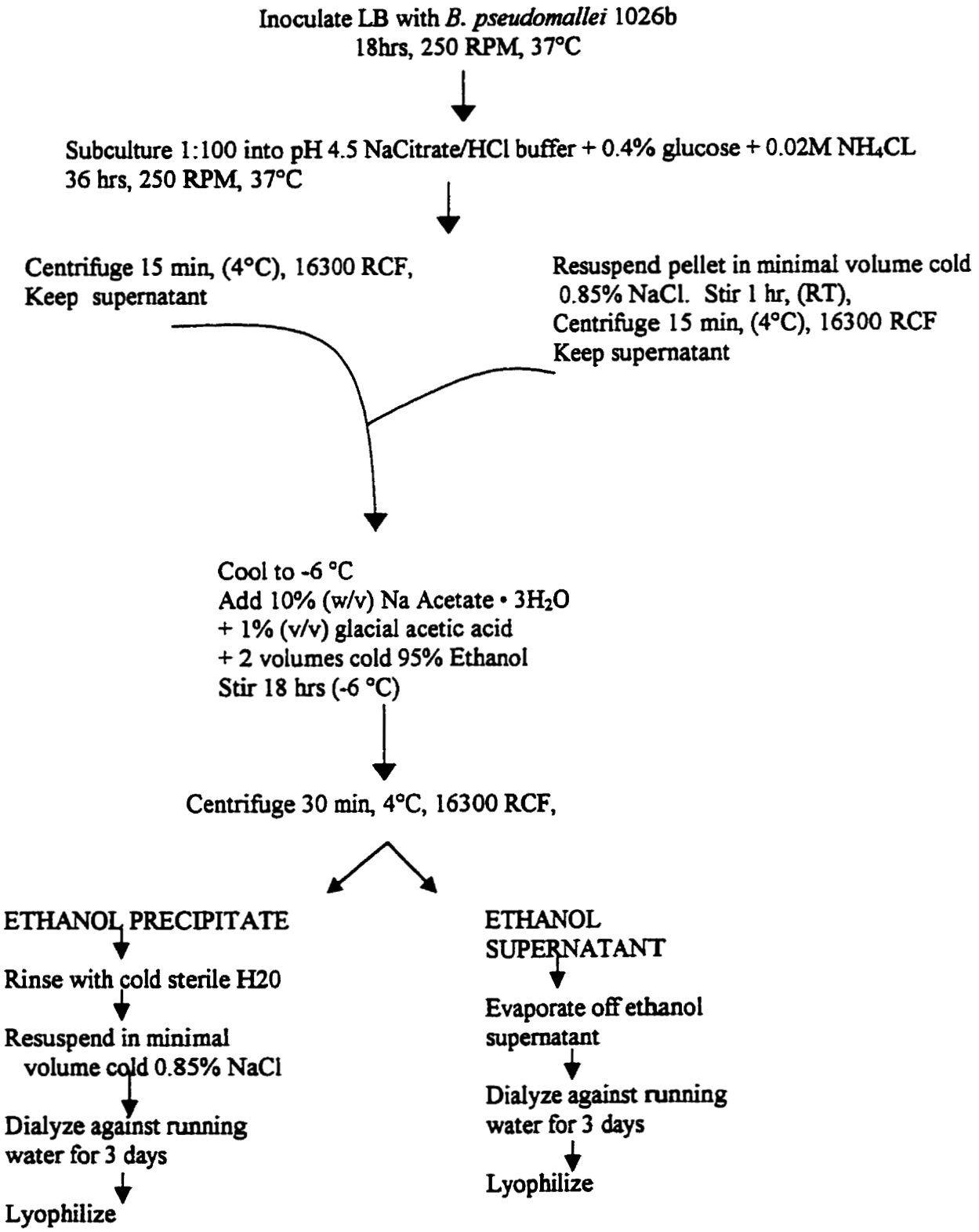
similar to the positive control, indicating approximately 2 mg/ml polysaccharide were present in each fraction.

The pooled column fractions were also assayed for the presence of any residual glucose from the growth media (Table 13). As a positive control, glucose (6.25%) was assayed concurrently with the pooled column fractions. In order to ensure that this assay did not detect any glucose moieties that could be components of the polysaccharide, two disaccharide controls were also included. Lactose (1 mg/ml), a disaccharide of glucose and galactose, and maltose (1 mg/ml), a disaccharide of glucose, were assayed concurrently with the pooled column fractions. With the exception of the positive control, there was essentially no glucose detected in the pooled column fractions or in the disaccharide samples.

KDO is present in both Type I LPS and Type II LPS as well as in the capsule. All pooled column fractions were assayed for the presence of KDO (Table 14). As a positive control, purified KDO (1 mg /ml) was concurrently assayed. There was no evidence of KDO in any pooled column fraction. Without the presence of KDO in the fractions, the isolated EPS should not be the previously documented capsule. While the absence of KDO in the EPS fractions should also indicate that it is not LPS, it is possible that either LPS O-antigen may have been isolated without the core component of the LPS molecule which includes the KDO.

N-acetylneuramic acid or sialic acid is a common component of many bacterial produced polysaccharides. All pooled column fractions were assayed for the presence of sialic acid (Table 15). As a positive control, purified sialic acid (1 mg/ml) was concurrently assayed. There was no evidence of sialic acid in any pooled column

Figure 17. *Burkholderia pseudomallei* 1026b Extracellular Polysaccharide Purification Protocol.



ETHANOL PRECIPITATE

↓

TCA precipitate proteins
Protein assay

↓

Treat with DNase I / RNase A

↓

Sepharose CL-4B Size
Exclusion Column

↓

Ethanol Precipitate Fractions
(EP fractions)

ETHANOL SUPERNATANT

↓

TCA precipitate proteins
Protein assay

↓

Sepharose CL-4B Size
Exclusion Column

↓

Rotovap Residue Fractions
(RVR fractions)

Table 12. Characterization of isolated exopolysaccharide fractions produced by *Burkholderia pseudomallei* 1026b at low pH by orcinol-sulphuric acid assay.[a]

Isolated Fraction	OD 405nm
Positive control (dextran sulphate 2mg/ml)	2.86
RVR 5A	2.52
RVR 5B	2.52
RVR 5C	2.59
EP 2A	2.27
EP 2B	2.49

The exopolysaccharide was isolated as described in Figure 17. The isolation procedure resulted in two sets of fractions. One set was isolated from the ethanol precipitate (called EP fractions), and the other set was isolated from residue after evaporating off the ethanol supernatant (called RVR fractions). After fractionation by a size exclusion column, each pooled fraction was assayed for the presence of polysaccharide.

[a] The orcinol-sulphuric acid assay measures the presence of total sugars.

Table 13. Measurement of residual glucose in exopolysaccharide fractions isolated from *Burkholderia pseudomallei* 1026b at low pH.

Isolated Fraction	OD 505nm
Positive control (6.25% glucose)	2.10
RVR 5A	0.04
RVR 5B	0.02
RVR 5C	0.02
EP 2A	0.01
EP 2B	0.01
Lactose (1mg/mL)[a]	0.01
Maltose (1mg/ml)[b]	0.01

The exopolysaccharide was isolated as described in Figure 17. The isolation procedure resulted in two sets of fractions. One set was isolated from the ethanol precipitate (called EP fractions), and the other set was isolated from residue after evaporating off the ethanol supernatant (called RVR fractions). After fractionation by a size exclusion column, the fractions were assayed to ensure that there was no residual glucose from the growth media present. In order to ensure that this assay did not detect any glucose moieties that could be components of the polysaccharide, two disaccharide controls were also included.

[a] Lactose is a disaccharide of glucose and galactose

[b] Maltose is a disaccharide of two glucoses

Table 14. Measurement of KDO in exopolysaccharide fractions isolated from *Burkholderia pseudomallei* 1026b at low pH.

Isolated Fraction	OD 548nm
Positive control (KDO 1mg/mL)	3.15
RVR 5A	0.04
RVR 5B	0.02
RVR 5C	0.03
EP 2A	-0.003
EP 2B	0.004

The exopolysaccharide was isolated as described in Figure 17. The isolation procedure resulted in two sets of fractions. One set was isolated from the ethanol precipitate (called EP fractions), and the other set was isolated from residue after evaporating off the ethanol supernatant (called RVR fractions). After fractionation by a size exclusion column, all fractions were assayed for the presence of KDO.

Table 15. Measurement of sialic acid in exopolysaccharide fractions isolated from *Burkholderia pseudomallei* 1026b at low pH.

Isolated Fraction	OD 549nm
Positive control (sialic acid 1mg/mL)	1.80
RVR 5A	0.02
RVR 5B	0.02
RVR 5C	0.05
EP 2A	0.02
EP 2B	0.03

The exopolysaccharide was isolated as described in Figure 17. The isolation procedure resulted in two sets of fractions. One set was isolated from the ethanol precipitate (called EP fractions), and the other set was isolated from residue after evaporating off the ethanol supernatant (called RVR fractions). After fractionation by a size exclusion, all fractions were assayed for the presence of sialic acid.

fraction.

3.4 Correlation of Exopolysaccharide Production at Acidic pH and Origin of *Burkholderia pseudomallei* Strains.

Dr. R. Finklestein (University of Missouri) graciously donated 220 *B. pseudomallei* strains collected in the 1960s. These include the laboratory strains Dr. Whitmore worked with in Burma, strains the Pasteur Institute isolated in Vietnam, as well as strains isolated from water in southern Thailand. Some of the south Thailand water strains were isolated from water with pH as low as two. These strains were all primary isolates. All 220 strains were assayed for EPS production at pH 4.5 in an attempt to correlate the strain origin with EPS production. Strains were assayed with orcinol-sulphuric acid to assess the amount of carbohydrate present after 24 hours growth in pH 4.5 sodium citrate buffer (0.4% glucose, 0.02 *M* NH_4Cl). Residual glucose from the growth media was quantitated. The total amount of EPS was calculated by subtracting the residual glucose from total carbohydrate produced. The strains were then grouped according to origin. There were 171 environmental strains and 49 laboratory strains. Ten clinical isolates and nine *B. thailandensis* strains were also assayed. Strains were categorized based on the amount of EPS produced at pH 4.5. Mucoid strains were defined as those producing greater than or equal to 400 μg EPS/ml. Weakly or non mucoid strains were defined as producing less than 400 μg EPS/ml. There was a highly significant difference between the number of mucoid and non mucoid strains in both the laboratory and environmental groups ($p<0.001$). The majority of environmental strains (70.2%) were mucoid while the majority of laboratory isolates (71.4%) were weakly or non mucoid (Table 16). There was also a significant difference between the number of

mucoid and non mucoid strains in the *B. thailandensis* group and the group of clinical isolates of *B. pseudomallei* ($p<0.01$). The majority of the clinical isolates (70%) were mucoid while without exception 100% of the *B. thailandensis* isolates were weakly or non mucoid. Both the laboratory and environmental strains demonstrated trends in the production of EPS (Figure 18). The majority of the laboratory strains produced between zero and 400 μg/ml EPS although there were stains that produced up to 6000 μg/ml EPS. The majority of the environmental strains produced higher amounts of EPS, between 400 and 3000 μg/ml EPS. There were environmental strains that produced as low as 68 μg/ml EPS and as high as 4400 μg/ml EPS. Overall, the trend was for environmental strains to produce higher amounts of EPS under acidic conditions. Some waters from which the environmental strains were isolated were reported to have pHs as acidic as two. It would be logical that these strains would undergo a type of natural selection in favor of mechanisms allowing for survival and possible proliferation in these extreme environments. Perhaps the EPS production is one such survival mechanism. It would also be logical for clinical isolates, which have been selected to survive in the hostile environmental of the host, would have evolved mechanisms to survive. In this case we hypothesize that the increased production of EPS in response to acidic conditions may have evolved in order to survive in the intracellular environment of a phagosome-lysosome.

Table 16. Correlation between the amount of extracellular polysaccharide produced at acidic pH and the origins of the *Burkholderia pseudomallei* strains.

	Number (%) of Mucoid strains [a]	Number (%) of Weakly or Non Mucoid strains [b]
Total number strains	141 (58.8)	98 (41.2)
Environmental strains	120 (70.2)	51 (29.8)
Laboratory strains	14 (28.6)	35 (71.4)
Clinical isolates	7 (70)	3 (30)
B. thailandensis strains	0 (0)	9 (100)

Overnight bacterial cultures were grown in LB broth and subcultured into pH 4.5 sodium citrate buffer. After 24 hours (37°C, 250 RPM), the cultures were assessed for amount of polysaccharide. Residual amounts of glucose were subtracted from the total amount of polysaccharide quantitated for each strain.

[a] Mucoid strains were defined as those able to produce ≥ 400ug EPS/ml after 24 hrs in pH 4.5 Na citrate/HCl buffer (37°C, 250 RPM).

[b] Weakly or non-mucoid strains were defined as producing < 400ug EPS/ml after 24 hrs in pH 4.5 Na citrate/HCl buffer (37°C, 250 RPM).

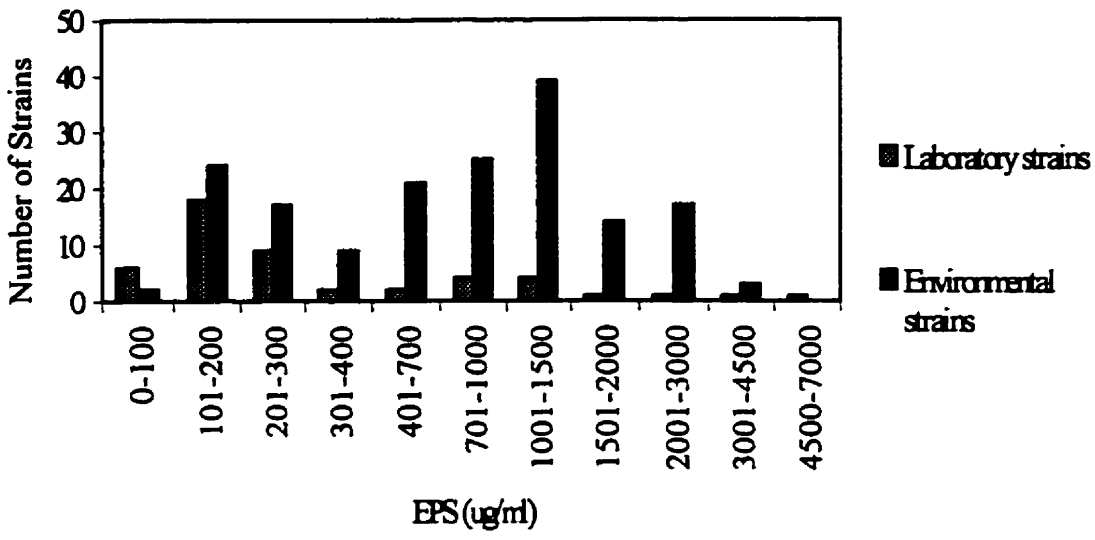


Figure 18. Correlation between the amount of extracellular polysaccharide produced at acidic pH and the origins of the *Burkholeria pseudomallei* strains. Overnight bacterial cultures were subcultured into pH 4.5 sodium citrate buffer. After 24 hours (37°C, 250 RPM), the cultures were assessed for amount of polysaccharide. Residual amounts of glucose were subtracted from the total amount of polysaccharide quantitated for each strain. The number of laboratory and environmental strains for each category of EPS mass was plotted in order to identify any trends.

4. DISCUSSION

Melioidosis has been termed "The Remarkable Imitator" because of its ability to mimic other diseases in its clinical presentation. It may manifest clinically as acute, subacute, chronic or latent illnesses. In the case of latent illness, the longest dormant period on record is the case of a Vietnam veteran who had a reactivation of the disease after an inapparent infection acquired in an endemic area 29 years earlier. Ribotype analysis has demonstrated that relapse of disease can result from a persistent source of endogenous infection. The recrudescence of this disease may be explained by the intracellular existence of *Burkholderia pseudomallei.* Once this pathogen has circumvented the intrinsic host defenses, it resides in a nutrient rich environment, safe from detection by the humoral arm of the immune system.

We have demonstrated that this pathogen is invasive at a MOI as low as one bacterium per eukaryotic cell. At that level, 0.2 % of the inoculum invaded the A549 monolayer. A549 type II pneumocytes are human alveolar carcinoma cells that possess the biochemical and morphological characteristics, including the multilamellar cytoplasmic inclusion bodies, of the type II pneumocytes from the intact lung.[114] As type II alveolar cells produce lung surfactant, and defects in either its production or secretion have been implicated in a variety of respiratory diseases, the ability of this pathogen to interact with an established pulmonary epithelial cell line allows investigation into the pulmonary pneumonia associated with melioidosis.[115] It has certainly enhanced our understanding of how *B. pseudomallei* invades and resides in the safe environs of the epithelial cell.

Burkholderia thailandensis was originally identified as a non-virulent form of *B. pseudomallei.* Due to a 15 nucleotide dissimilarity in the 16S ribosomal RNA genes, as

well as genotypic and phenotypic differences, it was reclassified as a distinct species. We have identified an additional difference between the two species. *B. thailandensis* E264 invaded A549 type II pneumocytes at a signficantly lower rate then *B. pseudomallei* 1026b ($p<0.001$). We showed that 1.83% of the *B. pseudomallei* inoculum invaded the monolayer while 0.04% of the initial *B. thailandensis* bacterial load invaded the A549 cells. An inability to establish a significant intracellular bacterial load would certainly hinder the virulence of *B. thailandensis.*

Many invasive pathogens, including *Shigella, Salmonella* and *Yersinia,* require the exploitation of host microfilaments (actin and myosin) for successful invasion. Previous studies have established that *B. pseudomallei* invasion is impeded when host microfilament rearrangement is inhibited.[49] We investigated whether the exploitation of host microtubules (tubulin) is essential for successful invasion by *B. pseudomallei.* The eukaryotic monolayer was pretreated with nocodazole (dissolved in DMSO), a specific inhibitor of microtubule rearrangement. As a control, a sample in which the monolayer was pretreated with DMSO alone was concurrently assayed. The results were inconclusive as to whether any of the demonstrated inhibition of invasion was due to the presence of the microtubule inhibitor or if it could all be accounted for by the presence of DMSO. *B. pseudomallei* invaded the untreated A549 monolayer at 1.83% of the inoculum. Invasion decreased to 0.7% of the inoculum in the presence of DMSO alone and 0.5% of the inoculum in the presence of nocodazole dissolved in DMSO. The difference in the invasion rates of the untreated and treated monolayers could be the result of the solvent in which the nocodazole is dissolved and not the presence of the microtubule inhibitor itself. Organisms that require the use of microtubules to invade

eukaryotic cells are often unable to replicate and persist within the host cells. This is clearly not consistent with the pathogenesis of *B. pseudomallei.* The lack of inhibition of invasion in the presence nocodazole is consistent with reports that other pathogens that require invasion as an essential virulence mechanism require the exploitation of microfilaments but not microtubules.

We have demonstrated that *B. pseudomallei* 1026b, a lethal clinical blood isolate, is able to invade A549 type II pneumocytes and successfully reside in that environment. Over 24 hours, this pathogen multiplied by 33 fold in the tissue culture monolayer compared to the basal number of intracellular microorganisms established at four hours. Using transmission electron microscopy, this bacterium was visualized residing in membrane bound vacuoles. The intracellular replication of this pathogen was also visualized inside A549 type II pneumocytes using electron microscopy. Recently, data were published confirming the intracellular replication of *B. pseudomallei* in RAW 264 macrophages and L929 fibroblast cells.[58] This report also stated there was preliminary evidence that *B. pseudomallei* could replicate in CHO cells, an epithelial cell line, although no data were presented. We have provided conclusive evidence demonstrating that this pathogen can indeed replicate in an epithelial cell line.

The sequence of events that follows the internalization of this pathogen has yet to be elucidated. We have demonstrated that in epithelial cells, one event that follows internalization is replication, generally in membrane bound vacuoles or phagosomes. We have investigated the occurrence of another event, the phagosome-lysosome fusion in epithelial cells. A recently published study was not been able to demonstrate a phagosome-lysosome fusion in macrophages using electron microscopy.[58] In our study,

the DNA sequence of the GFP protein from *Aequorea victoria* was successfully introduced into the genome of *B. pseudomallei* by conjugation. Invasion assays were performed, and it was established that the production of this protein did not affect the ability of *B. pseudomallei* to invade A549 cells. Using confocal microscopy, we investigated the occurrence of a phagosome-lysosome fusion. The lysosomes were labeled using the Lysotracker Red probe. The occurrence of a phagosome-lysosome fusion was established by the colocalization of fluorescence emitted by the lysosome and the pathogen. There are many intracellular pathogens that survive a phagosome-lysosome fusion. Survival generally involves some sort of modification of the vacuoles or the host lysosome. The mechanism by which *B. pseudomallei* survives the phagosome-lysosome fusion is, as of yet, completely unknown. Further studies are necessary to establish if the fusion is a complete or partial event. In the intracellular pathogenesis of *Salmonella typhimurium,* vacuoles containing this pathogen have lysosomal markers such as lysosomal glycoproteins but lack lysosomal enzymes including cathepsin D.[76] In this manner, *S. typhimurium* prevents a complete phagosome-lysosome fusion event, resulting in a successful infection. Pathogens may also prevent vacuole acidification, decrease the host oxidiative burst, develop resistance to the lysosomal contents or evolve to grow better at low pH in order to survive. It is not yet known which, if any, of these mechanisms *B. pseudomallei* employs.

The molecular basis of *B. pseudomallei* invasion is not well characterized. Through transposon mutagenesis and invasion assay screening, we have identified 28 mutants deficient in the invasion of A549 cells compared to the parental strain, *B.*

pseudomallei 1026b. Eighteen of these mutants contained transposon insertions in genes with identifiable DNA sequence homology.

Invasion of eukaryotic cells involves a significant amount of stress on the bacterial cell. An important part of host nonspecific immunity is nutritional immunity in which host cells attempt to withhold essential nutrients from invading pathogens. This results in the induction of many stress or starvation related genes. Among these are the genes of the phosphate regulon, which are induced under phosphate starvation conditions. The DNA flanking the tranposon insertion of one invasion deficient mutant demonstrated significant homology to the DNA sequence which encodes PhoB, a response regulator protein of a two-component regulatory system in *Haemophilus influenza, Pseudomonas aeruginosa, Shigella dysenteriae, E.coli,* and *Shigella flexneri.* This DNA sequence also demonstrated homology to the DNA sequence of response regulators in *Providencia stuardtii* and *Lactobacillus sakei,* as well as homology to the DNA sequence of phosphate regulatory proteins in *Sinorhizobium meliloti* and *Bradyrhizobium japonicum.* In *E. coli,* the phoB response regulator is part of a two component regulatory system that controls the induction of alkaline phosphatase (phoA), periplasmic phosphate binding proteins (phoS), an outer membrane porin (phoE), sn-glycerol-3-phosphate uptake (ugpAB), phosphate specific transport (pst) and phosphate starvation inducible genes (psiEO).[116] One report demonstrated that the deduced amino acid sequence of the *S. typhimurium* phoP protein had extensive homology to the *E. coli* phoB.[117] PhoP is a virulence gene transciptional regulator without which *S. typhimurium* becomes avirulent, unable to survive in macrophages and sensitive to antimicrobial peptides. Perhaps the phoB protein of *B. pseudomallei* may regulate virulence factors other than phosphate

starvation genes. Sequence analysis of the DNA flanking the tranposon insertion in a second invasion deficient mutant elucidated homology to the DNA sequence of the phoH protein in *E.coli* and *Thermotoga maritima.* The phoH protein has ATP binding affinity and is encoded for by phosphate starvation inducible genes.[118]

Another nutritive stress faced by bacterial cells is that of carbon starvation. We have identified one invasion deficient *B. pseudomallei* mutant in which the transposon has inactivated genes that share homology with the DNA sequence of carbon starvation proteins in *E. coli, Helicobacter pylori, Bacillus subtilis,* and *Mycobacterium tuberculosis.* The carbon starvation protein A (cstA) is an integral membrane protein that is involved in peptide utilization during carbon starvation.[119] This protein shares homology with a 77.9 KD protein in the MRR-TSR intergenic region which is also induced by carbon starvation. CstA is induced at the onset of carbon starvation and is positively regulated by cyclic AMP and the cyclic AMP receptor protein. In *S. typhimurium,* another gene under the positive control of the cyclic AMP system and induced by carbon limiting conditions is *fadF* which encodes an acyl-CoA dehydrogenase.[120] *FadF* is induced within Madin-Darby canine kidney epithelial cells suggesting that the induction of carbon starvation genes may be necessary for intracellular survival. In *S. typhimurium,* many genes that are induced under carbon starvation conditions are induced as a general stress-starvation response.[121] These genes can also be induced under phosphate or nitrogen limiting conditions, under extremes of pH or osmolarity or in the intracellular environment of epithelial cells. Finally the *Salmonella* plasmid-associated virulence genes required for systemic disease are inducible by carbon starvation.[121]

The cstA promoter region has been localized downstream of the iron regulated entCEBA-P15 operon which regulates the production of the iron chelator enterobactin in *E. coli.*[122] Iron limitation is a well-documented stress that bacterial pathogens must overcome both in the environment and during eukaryotic cell invasion, where iron is especially limited. Iron is important in the synthesis of DNA and RNA, electron transport, oxygen metabolism and nitrogen fixation.[123] Bacterial cells produce many secreted or cell surface products that enable them to scavenge iron in their chosen niche. Two invasion deficient *B. pseudomallei* mutants were identified with transposon insertions in genes with homologies to iron related genes. In one mutant, the homologous gene products were iron binding proteins in *Pasteurella haemolytica, Synechocystis spp, Neisseria gonorrhoeae, Neisseria meningitidis* and *Serratia marcescens,* iron transport proteins in *Synechocystsis spp.* as well as iron deficiency induced (idi) proteins in *Synechococcus spp.* The interrupted genes of the second invasion deficient mutant demonstrated homology to the DNA sequence of the pupIR proteins of *Pseudomonas putida* and the fecIR proteins of *E. coli.* Both are two component regulatory systems. The pup system[124] regulates siderophore production and the fec system is involved in the regulation of ferric dicitrate transport.[125] The fecI protein has a helix-turn-helix motif typical of DNA-binding regulatory proteins while fecR is a sensory protein that recognizes iron citrate.[125] Iron deprivation has been shown to affect adherence and invasion of certain bacterial pathogens. *N. gonorrhoeae,* in iron depleted media, was less able to adhere to human endometrial cells than in an iron rich media. The presence of transferrin in the tissue culture media increased the adherence of *N. gonorrhoeae* to human endometrial cells. In fact, in the presence of saturated human transferrin *N.*

gonorrhoeae was able to proliferate in the tissue culture media alone.[126] *Ehrlichia chaffeensis*, an obligate intracellular bacterium, is sensitive to intracytoplasmic iron concentration as its survival decreases in the presence of deferoxamine, an intracellular iron chelator.[127] *E. coli* invasion of brain microvascular endothelial cells is enhanced in iron supplemented media and repressed in the presence of iron chelators.[128] In *Listeria monocytogenes*, the *inlAB* virulence genes are positively regulated in iron rich environments.[129]

There is abundant evidence to suggest that functional stress and starvation inducible genes are necessary for successful infections by other bacterial pathogens. In our studies to characterize the genetics of *B. pseudomallei* invasion, we have shown this to be true as well. When mutations were established in a number of starvation inducible genes, *B. pseudomallei* was unable to produce a successful invasion of A549 type II pneumocytes. In some organisms, global regulatory mechanisms for the induction of virulence factors have been elucidated. Many virulence genes are coordinately regulated by a variety of environmental and genetic signals. *In vivo* induced (*ivi*) genes in *S. typhimurium* are coordinately regulated by low pH, low concentrations of iron or magnesium and the PhoPQ regulatory system.[91] The possibility of a similarly regulated system in *B. pseudomallei* seems evident based on the DNA sequence homologies that were elucidated for a number of invasion deficient mutants. Further studies in this area are especially important in order to elucidate the genetic regulation of invasion and begin development of appropriate prophylactic treatments.

Once a successful invasion has occurred in epithelial cells, this pathogen resides in a vacuole that undergoes a phagosome-lysosome fusion event. One microbicidal

weapon in the lysosomal array is the intracellular acid pH. In order to survive in this environment, a bacterial cell must be able to regulate its internal pH thus maintaining homeostasis. Studies into the mechanisms by which leukocytes maintain an intracellular pH have elucidated a cadmium dependent vacuolar type ATPase. The excess acid generated by the metabolic activity must be rapidly extruded in order to maintain normal microbicidal responses. Vacuolar ATPases are a family of proton pumps distributed from bacteria to higher organisms.[130] A. L. Jones characterized a *B. pseudomallei* invasion deficient mutant, AJ1D8, that was sensitive to cadmium, whereas the parental strain is resistant.[51] Using LB agar plates supplemented with 5 mM cadmium, we demonstrated that *B. pseudomallei* 1026b could grow in the presence of this cation, while AJ1D8 could not. The minimum concentration of cadmium necessary to inhibit the growth of *B. pseudomallei* 1026b was 9.6 mM, while to inhibit AJ1D8 growth, the necessary concentration was 0.6 mM cadmium.

Maintenance of cytoplasmic pH close to physiologic range is vital to normal cellular homeostasis. We used Oregon Green Dye to assess the intracellular pH of *B. pseudomallei* in sodium citrate buffers of pH 3, pH 5 or pH 7. This dye emits green fluorescence under acidic conditions. We hypothesized that *B. pseudomallei* may utilize a cadmium dependent vacuolar type ATPase to maintain a physiological intracellular pH under acid condition. We expected to see a proportion of the bacterial cells emitting fluorescence at pH 3 due to the turnover of protons in the cell. At pH 7, a neutral pH, there were virtually no bacterial cells with an acidic intracellular pH. When the pH of the buffer was decreased to pH 5, there was an increase in the number of bacterial cells with an acidic intracellular pH. Between four to five percent of the bacterial cells

demonstrated fluorescence. However, at pH 3, no bacterial cells were identified that emitted fluorescence. At pH 5 there was a proportion of the bacterial population whose proton efflux mechanisms had been overwhelmed. This resulted in an increase in the acidity of the cytoplasm as established by the low percentage of bacterial cells demonstrating intracellular fluorescence. It order for this dye to fluoresce, it requires acidic conditions and viable cell functions to enzymatically cleave the dye. It seemed improbable that in an environment with a higher proton concentration, the bacterial cells would be able to export all protons, resulting in a non-fluorescent bacterial population. Since fluorescence by this dye is dependent on active bacterial metabolic processes, we hypothesized that pH 3 may be bactericidal to *B. pseudomallei* 1026b.

B. pseudomallei was challenged by incubation at pH 3 in order to assess survival. Approximately 69% of the bacteria survived a 30 minute challenge. As previously mentioned, a vacuolar type ATPase that is cadmium dependent has been elucidated in leukocytes. Excess cadmium was added to the challenge media to see if its presence could ameliorate *B. pseudomallei* survival. In the presence of cadmium, survival increased to over 90%. Despite the fact that zinc has also been implicated in the function of vacuolar type ATPases and resistance to zinc is regulated by a two component system, czc, which links cadmium, zinc and copper, the presence of zinc in the challenge media was a detriment to *B. pseudomallei* survival at pH 3. Survival dropped to 35% in presence of zinc.

In order to continue studies on the physiological response of *B. pseudomallei* to acid conditions, the most acidic pH that supported optimal growth was elucidated. A graded pH series of sodium citrate buffers was assessed for optical density (600 nm) after

being subcultured from an overnight culture of *B. pseudomallei* grown in LB broth. Between pH 4.4 and pH 4.3, there was a dramatic decrease in optical density units, from 1.1136 to 0.1593. The most acidic pH that supported abundant bacterial growth was pH 4.5. That pH was selected for all further experiments.

One well documented bacterial response to acidic conditions is that of *H. pylori*. This gastrointestinal pathogen secretes a urease which acts as a buffer against the acid pH of the stomach. As the acid pH of the lysosome is a major bactericidal challenge to intracellular pathogens, urease production by *B. pseudomallei* was investigated. We speculated that *B. pseudomallei* may secrete a urease in response to the acidic challenge faced upon phagosome-lysosome fusion. There was no evidence that urease was produced in response to growth in pH 4.5 sodium citrate buffer. To assess the production of an inducible urease, this pathogen was also grown in M9 media containing urea as a sole source of nitrogen. In order to proliferate, the bacteria must produce a urease to cleave the urea and release nitrogen. After 24 hours, there was abundant bacterial growth as evidenced by visible turbidity. However, analysis using Bacto urea broth revealed no inducible urease. It appears that urease production is not a survival mechanism utilized by this pathogen. An interesting area of study will be to determine which mechanisms *B. pseudomallei* does utilize to survive in acidic pH conditions such as found in a phagosome-lysosome.

Once an optimal pH for bacterial growth had been established by optical density, the effect of growth in pH 4.5 sodium citrate buffer on this pathogen after 48 hours was investigated. At zero hours, *B. pseudomallei* had approximately the same cell number and optical density (600 nm) in LB broth or pH 4.5 sodium citrate buffer. After 48 hours

(250 RPM, 37°C), the optical density in both samples was similar, but there was a difference in cell number. Growth curves were then performed for *B. pseudomallei* in LB broth and pH 4.5 sodium citrate buffer. As expected there was a logarithmic increase in both the cell number and optical density (600 nm) of *B. pseudomallei* grown in LB broth. In the pH 4.5 sodium citrate buffer sample, there was a logarithmic increase in optical density but, at maximum, a doubling of cell number. Over 48 hours there was an increase in optical density that could not be accounted for by a corresponding increase in cell number. Using India ink, we visualized the bacteria after growth in LB and in pH 4.5 sodium citrate/HCl buffer (data not shown). There was no evidence of cell elongation to account for the increase in optical density.

We postulated that the increase in optical density observed when *B. pseudomallei* was grown in pH 4.5 conditions was due to the secretion of an exopolysaccharide. We used the orcinol-sulphuric acid assay to investigate our hypothesis. In this assay, concentrated sulphuric acid causes the hydrolysis of all glycosidic linkages in the polysaccharide and the subsequent dehydration product reacts with the orcinol to give a coloured endpoint. A dextran standard curve was assayed concurrently with all samples in order to quantitate the results. At 24 hours, there was an increase in the amount of EPS in both the whole cell and supernatant fractions of the *B. pseudomallei* sample grown in pH 4.5 sodium citrate buffer compared to the samples grown in LB broth. There was eight times more EPS in the whole cell fraction and nine times more EPS in the supernatant. At 48 hours, there was five times more EPS in the whole cell fraction and four times more EPS in the supernatant of the pH 4.5 sample compared to the LB sample.

After 48 hours, some cells may lyse, releasing degradative enzymes. This would account for the apparent decrease in EPS at 48 hours compared to 24 hours.

Three cell surface components of *B. pseudomallei* have been well characterized. LPS Type I has an O-antigen composed of 2-O acetyl-6-deoxy manno-heptose repeats. The O-antigen of LPS Type II is a D-glucose β-1,3-6 deoxy-L-talose repeat. The third cell surface component that has been characterized is a capsule composed of three galactoside residues with a KDO moiety and an O-acetyl group at the 2-position. Using antibodies to these cell surface components, we investigated whether the increase in EPS that *B. pseudomallei* produced at pH 4.5 was the result of increases in one of these characterized cell surface saccharides. Using ELISAs, we established that there was not an increase in the amount of capsule produced when *B. pseudomallei* was grown at pH 4.5. We elucidated a slight increase in the amount of LPS Type-I and an increase in the amount of LPS Type-II at pH 4.5. It appears that the increase in optical density, corresponding to an increase in EPS, that occurs when *B. pseudomallei* in grown in pH 4.5 sodium citrate buffer may be partially accounted for by an increase in LPS.

We attempted to isolate the putative EPS using the methods of Alms and Bass[111] which involves an ethanol precipitation step (Figure 17). We discovered that there was polysaccharide remaining in the ethanol after the precipitation step. The EPS in this fraction may have been too small to be precipitated out with ethanol This resulted in two sets of polysaccharide fractions. One set was isolated from the ethanol precipitate (EP) and one set was isolated from the ethanol by evaporation (RVR). After being fractionated on a size exclusion column, five fractions were characterized.

The pooled fractions were assayed to confirm the presence of EPS. All fractions demonstrated an orcinol reaction comparable to that of dextran sulphate at 2 mg/ml. No residual glucose from the growth media was detected in any samples. In order to ensure that this assay did not detect glucose residues that could be a component of the putative EPS, disaccharides containing glucose were also assayed. There was no glucose reaction detected when either lactose (disaccharide of glucose and galactose) or maltose (disaccharide of glucoses) was assayed.

KDO is present in many different bacterial polysaccharides including the capsule and both LPS moieties of *B. pseudomallei*. All fractions were assayed for the presence of KDO with purified KDO assayed concurrently as a positive control. There was no evidence of KDO in any fraction. Without the presence of KDO in the fractions, this putative EPS could be an entirely novel cell surface component. Conversely, the isolated EPS may be the O-PS from either LPS molecule without any core components present.

N-acetylneuramic acid or sialic acid is a common component of many bacterial produced polysaccharides. The capsulated strains of Group B *Streptococci* that contain sialic acid cause a septic arthritis that is not seen to the same extent in those strains without sialic acid as a part of the capsule. The sialic acid residues influence the incidence of articular lesions seen in septic arthritis. The increased virulence of group III-3 GBS over group III-2 has been attributed to its higher sialic acid content. Sialic acid is believed to inhibit opsonophagocytosis and C5a production. There are terminal sialic acid residues in the lipooligosaccharides of *Haemophilus ducreyi* and *Corynebacterium diptheriae*. The sialylated LOS of *N. gonorrhae* mediates serum resistance in that pathogen, and in *P. aeruginosa*, sialic acid mediates *in vivo* adherence. There was no

evidence of sialic acid in any fractions of the isolated putative EPS produced by *B. pseudomallei* at pH 4.5.

Is the increase in optical density in *B. pseudomallei* cultures grown at pH 4.5, corresponding to an increase in EPS, a constitutive response to acid conditions by all strains of *B. pseudomallei*? Using 220 strains of *B. pseudomallei* graciously donated by Dr. R. Finklestein, we addressed this question by assaying the amount of EPS produced by all strains at pH 4.5. Strains were grouped as laboratory or environmental strains in order to correlated EPS production with strain origin. Mucoid strains were defined as producing greater than or equal to 400 μg/ml EPS while non mucoid or weakly mucoid strains were defined as producing less than 400 μg/ml EPS. There was a highly significant difference in the number of mucoid and non mucoid strains in both groups ($p<0.001$). The majority of the environmental strains, 70.2%, were mucoid while the majority of the laboratory strains, 71.4%, were weakly or non-mucoid. Both the environmental and laboratory groups of *B. pseudomallei* strains demonstrated a trend in EPS production. There was a bell curve that represented each group whereby the majority of strains peaked at the same amount of EPS although there were representatives of each group throughout the entire spectrum of EPS mass. The majority of the environmental strains, which produced higher amounts of EPS than the laboratory strains, produced a bell curve of EPS that peaked at 1000 to 1500 μg/ml. The majority of the laboratory strains, which produced lower amounts of EPS than the environmental strains, produced a bell curve of EPS that peaked at 100 to 200 μg/ml. Interestingly, it was a laboratory strain that produced the highest amount of EPS, over 6000μg/ml. Overall, the trend seems to be for environmental strains to produce higher amounts of EPS under

acidic conditions. Environmental strains undergo a different set of selective pressures in order to proliferate. *B. pseudomallei* has been isolated in waters off southern Thailand where the reported pH was as low as two. It would be logical that these strains would undergo a more stringent natural selection in favor of mechanisms allowing for survival under extreme circumstances. Increased EPS production under acidic conditions may act as a physical barrier preventing proton access to the bacterial cells or it may neutralize the acidity of the media.

There was also a highly significant difference between the number of mucoid and non mucoid strains in the *B. pseudomallei* clinical isolate group as compared to the *B. thailandensis* group ($p<0.01$). As with the environmental group, the clinical strains have evolved to survive in a highly specialized niche. There are many intrinsic host defenses that an intracellular pathogen must be able to overcome in order to establish a successful endogenous infection. We hypothesize that the ability of clinical isolates of *B. pseudomallei* to survive in the acidic environment of a phagosome-lysosome fusion is due to the increased production of EPS. The closely related *B. thailandensis* strains were all weakly muoid, suggesting that increased production of EPS may be an acquired virulence factor specifically for host infection and not necessarily environmental survival as *B. thailandensis* is an environmental organism. This bacterial EPS may provide a protective envelope for the invading pathogen.

In our studies into the intracellular existence of *B. pseudomallei*, we have elucidated a number of relevant observations about the pathogenesis of disease due to this organism. We have demonstrated that *B. pseudomallei* is significantly more invasive than *B. thailandensis*. We have demonstrated that the invasion of this pathogen is not

dependant on host microtubule exploitation. Once internalization has occurred, this pathogen can replicate in the intracellular milieu and withstand a phagosome fusion. Using tranposon mutagenesis, we showed that invasion deficient mutants had interruptions in DNA sequences related to stress or starvation responses and hypothesized that these responses may be components of a global regulation system. As part of the physiological response to acidic pH, we have shown that *B. pseudomallei* produces an extracellular polysaccharide. Studies are currently underway to identify the components of that polysaccharide.

We have initiated preliminary studies into the above mentioned areas but further investigations are necessary. As our evidence is the first to demonstrate a phagosome-lysosome fusion as part of the events that occur once *B. pseudomallei* has been internalized, there are many areas that need to be investigated. It is necessary to establish if a full or partial fusion event occurs and if this pathogen remains in the fused vacuole or escapes to the cytoplasm. It should also be established if this pathogen can temper the events that occur in the lysosome, such as modulating the oxidative burst. Another area of interest is if the bacteria stay in epithelial cells or transcytose for deeper invasion.

We have elucidated several stress and starvation responses are necessary for *B. pseudomallei* to successfully invade A549 type II pneumocytes. Further studies are necessary to determine if these responses are under the control of a global regulatory system.

We have demonstrated that *B. pseudomallei* produces an EPS in response to an acidic challenge. Once we have established the monsaccharide constituents of the EPS that was isolated from *B. pseudomallei* grown in pH 4.5 conditions, the immune response

to the purified EPS should be established to see if it can elicit protection against *B. pseudomallei* infection. The constitutive expression of this EPS should be investigated using antibodies raised to this EPS. These investigations could potentially lead to new and exciting vaccine therapeutics.

The purpose of this thesis was to continue investigations into the intracellular existence of *B. pseudomallei*. This is an exciting area of expanding interests. Our findings should provide valuable insight into the pathogenesis of disease due to this pathogen.

BIBLIOGRAPHY

[1] Whitmore, A., & Krishnaswami, C.S. (1912) An account of the discovery of a hitherto undescribed infective disease occurring among the population of Rangoon. *Ind. Med. Gazette*. **47**:262-267.

[2] Sanford, J.P. (1990) Pseudomas species (including melioidosis and glanders). In Principlas and Practice of Infectious Diseases, 3rd ed. pp. 1692-1696. Ed. Mandell, G.L., Douglas Jr, R.G. & Bennet, J.E. New York: Churchill Livingstone.

[3] Howe, C., Sampath, A. & Spotnitz, M. (1971) The *Pseudomallei* group: A review. *J. Inf. Dis.* **124**(6):598-606.

[4] Nigg, C. (1963) Serological studies on subclinical melioidosis. *J. Immun.* **91**:18-28.

[5] Sponitz, M., Rudnitzky, J. & Rambaud, J.J. (1967) Melioidosis pneumonitis. *J.A.M.A* **202**:950-995.

[6] Poe, R.H, Vassallo, C.L, & Domm, B.M. (1971) Melioidosis: The remarkable imitator. *Rev. Resp. Dis. Am.* 104:427-431.

[7] Mays, E.E., & Rickets, E.A. (1975) Melioidosis: Recrudescence associated with bronchogenic carcinoma twenty-six years following initial geographic exposure. *Chest* **68**(2):261-262.

[8] Chodimella, U, Hoppes, W.L, Whalen, S, Ognibene, A. & Rutecki, G.W. (1997) Septicemia and suppuration in a vietnam veteran. *Hosp Pract. (Off Ed)* **32**(5):219-221.

[9] Yabuuchi, E. & Arakawa, M. (1993) *Burkholderia pseudomallei* and melioidosis: Be aware in temperate area. *Microbiol. Immunol.* **37**(11):823-836.

[10] Dance, D.A.B. (1991) Melioidosis: The tip of the iceberg. *J. Clin. Microbiol.* **4**(1):32-60.

[11] Chaowagul, W. (1996) Melioidosis: A treatment challenge. *Scand. J. Infect. Dis. Suppl* **101**:14-16.

[12] Chaowagul, W., suputtamongkol, Y., Dance, D.A.B, Rajchanuvong, A. Pattara-arechachai, J. & White, N.J. (1993) Relapse in melioidosis: Incidience and risk factors. *J. Inf. Dis.* **168(5)**:1181-1185.

[13] McCormick, J.B, Sexton, D.J, McMurray, J.G, Carey, E., Hayes, P. and Feldman, R.A. (1975) Human-to-human transmission *of Pseudomonas pseudomallei. Ann. Int. Med.* **83**:512-513.

[14] Green, R.N. & Tuffnell, P.G. (1968) Laboratory acquired melioidosis. *Am. J. Med.* **44**:599-605.

[15] Schlech, W.F, Turchik, J.B., Westlake, R.E., Klein, G.C., Band, J.D. & Weaver, R.E. (1981) Laboratory-acquired infection with *Pseudomonas pseudomallei* (melioidosis). *New Eng. J. Med.* 305(19):11331135.

[16] Ashdown L.R. (1979) An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens. *Pathology*. **11**:293-297.

[17] Brett, P.J., DeShazer, D. & Woods. D.E. (1997) Characterization of *Burkholderia pseudomallei* and *Burkholderia pseudomallei*-like strains. *Epidemiol. Infect.* **118**:137-148.

[18] Brett, P.J., DeShazer, D. & Woods, D.E. (1998) *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. *Internat'l J. Sys. Bac.* **48**:317-320.

[19] Dannenberg Jr Am. Scott E. (1957) Melioidosis: Pathogenesis and immunity in mice and hamsters. I. Studies with virulent strains of *Malleomyces pseudomallei. J. Exp. Med.* 107:153-187.

[20] Dannenberg Jr Am. Scott E. (1958) Melioidosis: Pathogenesis and immunity in mice and hamsters. II. Studies with avirulent strains of *Malleomyces pseudomallei. Am. J. Pathol.* **34**:1099 -1121.

[21] Smith, M.D., Angus, B.J., Wuthiekanun, V. & White, N.J. (1997) Arabinose assimilation defines a nonvirulent biotype *of Burkholderia pseudomallei. Infec. Immun.* **65(10):**4319-4321.

[22] Arakawa, M. & Yabuuchi, E. (1991) *Psuedomonas pseudomallei. Antibiot. Chemother.* 7:1121-1127.

[23] Brett, P.J., Mah, D.C.W. & Woods, D.E. (1994) Isolation and characterization and *Pseudomonas pseudomallei* flagellin proteins. *Infec. Immun.* **62(5):**1914-1919.

[24] DeShazer, D., Brett, P.J., Carlyon, R. & Woods, D.E. (1997) Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: Isolation of motility mutants and molecular characterization of the flagellin structural gene. *J. Bac.* **179(7):**2116-2125.

[25] Tong, S., Yang, S., Lu, Z. & He, W. (1996) Laboratory investigation of ecological factors influencing the environmental presence of *Burkholderia pseudomallei. Microbiol. Immunol.* **40(6)** :451-453.

[26] Wuthiekanun, V., Smith, M.D., & White, N.J. (1995) Survival of *Burkholderia pseudomallei* in the absence of nutrients. *Trans. Roy. Soc. Trop. Med & Hyg.* **89**:491.

[27] Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. & Arakawa, M. (1992) Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. now. *Microbiol. Immunol.* **36**(12) 1251-1275.

[28] Mandel, M. (1966) Deoxyribonucleic acid base composition in the genus *Pseudomonas. J. Gen. Microbiol.* **43**:273-292.

[29] Lew, A.E., & Desmarchelier, P.M. (1993) Molecular typing of *Pseudomonas pseudomallei*: restriction fragment length polymorphisms of rRNA genes. *J. Clin. Microbiol.* **31**:533-539.

[30] Sexton, M.M., Goebel, L.A., Godfrey, A.J.,Choawagul, W., White, N.J. & Woods, D.E. (1993) Ribotype analysis of *Pseudomonas pseudomallei* isolates. *J. Clin. Microbiol.* **31**(2):238-243.

[31] Trakulsomboon, S., Dance, D.A.B.,Smith, M.D., White, N.J. & Pitt, T.L. (1997) Ribotype differences between clinical and environmental isolates of *Burkholderia pseudomallei. J. Med. Microbiol.* **46**:565-570.

[32] Haase, A., Smith-Vaughan, H., Melder, A., Wood, Y., Janmaat, A., Gilfedder, J., Gilfedder, J., Kemp, D. & Currie, B. (1995) Subdivision of *Burkholderia pseudomallei* ribotypes into multiple types by random amplified polymorphic DNA analysis provides new insights into epidemiology. *J. Clin. Microbiol.* **33(7):**1687-1690.

[33] Rapaport, F.T., Miller, J.W. & Ruch, J. (1961) Endotoxic properties of *Pseudomonas pseudomallei. Arch. Pathol.* **71**:429-436.

[34] Colling, M., Nigg, C. & Heckly, R.J. (1958) Toxin of *Pseudomonas pseudomallei*. I. Production *in vitro. J. Bacteriol.* **76**:422-426.

[35] Haase, A., Janze, J., Barrett, S., & Currie, B. (1997) Toxin production by *Burkholderia pseudomallei* strains and correlation with severity of melioidosis. *J.Med. Microbiol.* **46**:557-563.

[36] Sexton, M.M., Jones, A.L., Chaowagul, W. & Woods, D.E. (1994) Purification and characterization of a protease from *Pseudomonas pseudomallei. Can. J. Microbiol.* **40**:903-910.

[37] Yang, H., Chaowagul, W. & Sokol, P.A. (1991) Siderophore production by *Pseudomonas pseudomallei. Infect. Immun.* **59**(3):776-780.

[38] Ashdown, L.R. & Koehler, J.M. (1990) Production of hemolysin and other extracellular enzymes by clinical isolates of *Pseudomonas pseudomallei. J. Clin. Micrbiol.* **28(10)**:2331-2334.

[39] Esselmann, M.T. & Liu, P.V. (1961) Lecithinase production by gram-negative bacteria. *J. Bacteriol.* **81**:939-945.

[40] Knirel, Y.A., Paramonov, N.A., Shashkov, A.S., Kochetkov, N.K., Yarullin, R.G., Farber, S.M. & Efremenko, V.I. (1992) Structure of the polysaccharide chain of *Pseudomonas pseudomallei* lipopolysaccharides. *Carbohy. Res.* **233**:185-193.

[41] Perry, M.B., MacLean, L.L., Schollaardt, T., Bryan, L.E. & Ho, M. (1995) Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. *Infect. & Immun.* **63(9)**:3348-3352.

[42] DeShazer, D., Brett, P.J. & Woods. D.E (1998) The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. *Molec. Microbiol.* **30(5)**:1081-1101.

[43] Brett, P.J. & Woods. D.E. (1996) Structural and immunological characterization of *Burkholderia pseudomallei* O-polysaccharide-flagellin protein conjugates. *Infec. & Immun.* **64**(7)2824-2828.

[44] Steinmetz, I., Rohde, M. & Brenneke, B. (1995) Purification and characterization of an exopolysaccharide of *Burkholderia (Pseudomonas) pseudomallei. Infect. & Immun.* **63(10)**: 3959-3965.

[45] Masoud, H., Ho, M., Schollaardt, T., & Perry, M.B. (1997) Characterization of the capsular polysaccharide of *Burkholderia (Pseudomonas) pseudomallei 304b. J. Bac.* **179(18)** :5663-5669.

[46] Nimtz, M., Wray, V., Domke, T., Brenneke, B., Haussler, S. & Steinmetz, I. (1997) Structure of an acidic exopolysaccharide of *Burkholderia pseudomallei Eur. J. Biochem.* **250**:608-616.

[47] DeShazer, D., Brett, P.J., Burtnick, M.N. & Woods. D.E. (1999) Molecular characterization of the genetic loci required for secretion of exoproducts in *Burkholderia pseudomallei. J. Bac.* **181(15)** 4661-4664.

[48] Woods, D.E., DeShazer, D., Moore, R.A., Brett, P.J., Burtnick, M.N., Reckseidler, S.L., & Senkiw, M.D. (1999) Current studies of the pathogenesis of melioidosis. *Microbes & Infection* **2**:157-162.

[49] Jones, A.L., Beveridge, T.J. & Woods, D.E. (1996) Intracellular survival of *Burkholderia pseudomallei. Infect. Immun.* **64**(3):782-790.

[50] Harley, V.S., Dance, D.A.B., Drasar, B.S. & Tovey, G. (1998) Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotice cells tissue culture. *Microbes* **96**:71-93.

[51] Jones, A.L., DeShazer, D. and Woods, D.E. (1997) Identification and characterization of a two-component regulatory system involved in invasion of eukaryotic cells and heavy-metal resistance in *Burkholderia pseudomallei. Infect. Immun.* **65**(12):4972-4977.

[52] Santanirand, P., Harley, V.S., Dance, D.A.B., Drasar, B.S. and Bancroft, G.J. (1999) Obligatory role of gamma interferon for host survival in a murine model of infection with *Burkholderia pseudomallei. Infec. Immun.* **67**(7):3593-3600.

[53] Miyagi, K., Kawakami, K. and Siato, A. Role of reactive nitrogen and oxygen intermediates in gamma interferon-stimulated murine macrophage bactericidal activity against *Burkholderia pseudomallei. Infec. Immun.* **65**(10):4108-4113.

[54] Ismail, G., Razak, N., Mohamed, R., Embi, N. and Omar, O. (1988) Resistance of *Pseudomonas pseudomallei* to normal human serum bactericidal action. *Microbiol. Immunol.* **32**(7):645-652.

[55] Harley, V.S., Dance, D.A.B., Tovey, G. & Drasar, B.S. (1994) Interaction of *Pseudomonas pseudomallei* with macrophages. *Biochem. Soc. Trans.* **22**:88s.

[56] Kishimoto, R.A. & Eveland, W.C. (1976) Interaction between *Pseudomonas pseudomallei* and cultured rabbit peritoneal macrophages. *Can. J. Microbiol.* **22**:1307-1311.

[57] Pruksachartvuthi, S., Aswapokee, N. & Thankerngpol, K. (1990) Survival of *Pseudomonas pseudomallei* in human phagocytes. *J. Med. Microbiol.* **31**:109-114.

[58] Harley, V.S., Dance, D.A.B., Tovey, G., McCrossan, M.V. and Drasar, B.S. (1998) An ultrastructural study of the phagocytosis of *Burkholderia pseudomallei. Micobios* **94**:35-45.

[59] Vorachit, M., Lam, K., Jayanetra, P. and Costerton, J.W. (1995) Electron microscopy study of the mode of growth of *Pseudomonas pseudomallei in vitro* and *in vivo J. Trop. Med. Hyg.* **98**:379-391.

[60] Egan, A.M. and Gordon, D.L. (1996) *Burkholderia pseudomallei* activates complement and is ingested but not killed by polymorphonuclear leaukocytes. *Infec. Immun.* **64**(12):4952-4959.

[61] Asam, T., Arpin, M., Prevost, C., Gounon, P. and Sansonetti, P.J. (1995) Cytoskeletal rearrangements and the function role of T-plastin during entry of *Shigella flexneri* into HeLa cells. *J. Cell. Biol.* **129**:361-387.

[62] Sansonetti, P.J., Ryter, A., Clerc, P., Maurelli, A.T. and Mounier, J. (1986) Multiplication of *Shigella flexneri* within HeLa cells:lysis of the phagocytic vacuole and plasmid mediated contact hemolysis. *Infec. Immun.* **51**:461-469.

[63] Maurelli, A.T., Baudry, B., dHauteville, H., Hale, T.L. and Sansonetti, P.J. (1985) Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri. Infec. Immun.* **49:164-171.**

[64] Sakai, T., Sasakawa, C., Makino, S. and Yoshikawa, M. (1986) DNA sequence and product analysis of the *virF* locus responsible for congo red binding and cell invasion in *Shigella flexneri 2a. Infect. Immun.* **54**:395-402.

[65] Watarai, M., Funato, S. and Sasakawa, S. (1996) Interaction of ipa proteins of *Shiglla flexneri* with alpha(5)beta(1) integrin promotes entry of the bacteria into mammalian cells. *J. Exp. Med* **183**:991-999.

[66] Takeuchi, A. (1967) Electron microscope studies of experimental Salmonella infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium. Am. J. Pathol.* **50:**109-136.

[67] Galan, J.E. (1996) Molecular genetic bases of *Salmonella* entry into host cells. *Mol. Microbiol.* **20:**163-271.

[68] Galan, J.E., Ginocchio, C. and Costeas, P. (1992) Molecular and functional characterization of the *Salmonalla* invasion gene *invA:* Homology of InvA to members of a new protein family. *J. Bacteriol.* **174:**4338-4349.

[69] Une, T. (1977) Studies on the pathogenicity of *Yersinia enterocolitica.* I. Experimental infection in rabbits. *Microbiol.Immunol.* **21:**341-363.

[70] Miller, V.L., Finlay, B.B. and Falkow, S. (1988) Factors essential for the penetration of mammalian cells by *Yersinia. Curr. Top. Microbiol. Immunol.* B138:15-39.

[71] Isberg, R.R. and Leong, J.M. (1990) Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. *Cell.* **60:**861-871.

[72] Miller, V.L. and Falkow, S. (1988) Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. *Infect. Immun.* **56:**1242-1248.

[73] Schultze-Koops, H., Burkhardt, H., Heesemann, J., Kirsch, T., Swobadoa, B., Bull, C., Goodman, S. and Emmrich, F. (1993) Outer membrane protein YadA on enteropathogenic yersiniae mediates specific binding to cellular but not plasma fibronectin. *Infect. Immun.* **61:**2513-2519.

[74] Finlay, B.B. and Falkow, S. Common themes in microbial pathogenicity revisited. *Micriobiol. Mol. Biol. Rev.* **61**(2):136-169.

[75] Rathman, M., Sjaastad, M.D. and Falkow, S. (1996) Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. *Infect. Immun.* **64**:2765-2773.

[76] Garcia-del Portillo, F. (1996) Interaction of *Salmonella* with lysosomes of eukaryotic cells. *Microbiologia SEM* **12**:259-266.

[77] Miller, S.I. (1991) PhoP PhoQ: macrophage-specific modulators of Salmonella virulence? *Mol. Microbiol.* **5**:2073-2078.

[78] Parra-Lopez, C., Lin, R., Aspedon, A. and Groisman, E.A. (1994) A *Salmonella* protein that is required for resistance to antimicrobial peptides and transport of potassium. EMBO J. **13**:3964-3972.

[79] Holtzman, E. (1989) Lysosomes p1-27,93,96,101,103-104,197-213,309. *In* Siekevitz, P. (series ed.) Cellular Organelles Plenum Press, New York, N.Y.

[80] Gabay, J.E., Horwitz, M.A. and Cohn, Z.A. (1986) Phagosome-lysosome fusion. *Biochem. Soc. Trans.* **14**:256-257.

[81] Roberts, I.S. (1996) The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu. Rev. Microbiol.* **50**:285-315.

[82] May, B.T. and Chakrabarty, A.M. (1994) *Pseudomonas aeruginosa:* genes and enzymes of alginate synthesis. *Trends Micriobiol.* **2**:151-156.

[83] Terry, J.M., Pina, S.E. and Mattingly, S.J. (1991) Environmental conditions which influence mucoid conversion in *Pseudomonas aeruginosa* PAO1. *Infect. Immun.* **59**(2):471-477.

[84] Terry, J.M., Pina, S.E. and Mattingly, S.J. (1992) Role of energy metabolism in conversion of nonmucoid *Pseudomonas aeruginosa* to the mucoid phenotype. *Infect. Immun.* **60**(4):1329-1335.

[85] Schlictman, D., Karanaugh-Black, A., Shankar, S. and Chakrabarty, A.M. (1994) Energy metabolism and alginate biosynthesis in *Pseudomonas aeruginosa:* role of the tricarboxylic acid cycle. *J. Bac.* **176**(19):6023-6029.

[86] Herbert, S., Worlitzsch, D., Dassy, B., Boutonnier, A., Fournier, J., Bellon, G., Dalhoff, A. and Doring, G. (1997) Regulation of *Staphylococcus aureus* capsular polysaccharide type 5: CO_2 inhibition in vitro and in vivo. *J. Inf. Dis.* **176**:431-438.

[87] Breedveld, M.W., Zevenhuizen, L.P.T., and Zehnder, A.J.B. (1990) Osmotically induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* SU-47. *J. Gen. Microbiol.* **136**:2511-2519.

[88] Zhan, H., Lee, C.C. and Leigh, J.A. (1991) Induction of the second exopolysaccharide (EPSb) in *Rhizobium meliloti* SU47 by low phosphate concentrations. *J. Bac.* **173**(22):7391-7394.

[89] Bortolussi, R., Ferrieri, P. and Quie, P.G. (1983) Influence of growth temperature of *Escherichia coli* on capsular antigen production and resistance to opsonization. *Infect. Immun.* **39**:1136-1141.

[90] Junkins, A.D. and Doyle, M.P. (1992) Demonstration of exopolysaccharide production by enterohemorrhagic *Escherichia coli. Curr. Microbiol.* **25**:9-17.

[91] Heithoff, D.M., Conner, C.P., Hentschel, U., Govantes, F., Hanna, P.C. and Mahan, M.J. (1999) Coordinate intracellular expression of *Salmonella* genes induced during infection. *J. Bac.* **181**(3):799-807.

[92] Soncini, F. and Groisman, E.A. (1996) Two-component regulatory systems can interact to process multiple environmental signals. *J. Bac.* **178**(23):6796-6801.

[93] Schuhmacher, D.A. and Klose, K.E. (1999) Environmental signals modulate ToxT-dependent virulence factor expression in *Vibrio cholerae. J. Bac.* **181**(5):1508-1514.

[94] Melton, A.R. and Weiss, A.A. (1989) Environmental regulation of expression of virulence determinants in *Bordetella pertussis. J. Bac.* **171**(11):6206-6212.

[95] Simon, R., Priefer, U., and Puhler, A. (1983) A broad host range mobilization system in *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *BioTechn.* **1**:784-791.

[96] Miller, V.L. and Mekalanos, J.J. (1988) A novel suicide vector and its use in construction of insertion mutations: Osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* require *toxR. J. Bac.* **170**:2575-2583.

[97] Boyer, H.W. and Roulland-Dussoix, D. (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli. J. Mol. Biol.* **41**:459-472.

[98] Merriman, T.R. and Lamont, I.L. (1993) Construction and use of a self cloning promoter and probe vector for gram negative bacteria. *Gene* **126**:17-23.

[99] Schweizer, H.P., Klassen, T. and Hoang, T. (1996) Improved methods for gene analysis and expression in *Pseudomonas spp.*, p.229-237. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), Molecular biology of pseudomonads. ASM Press, Washington, D.C.

[100] Cormack, B.P, Valdiva, R.H. and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**(1SpecNo):33-38.

[101] Elsinghorst, E.A. (1994) Measurement of invasion by gentamycin resistance. *Meth. Enz.* **236**:405-420.

[102] Rosenshine, I., Ruschkowski, S. and Finlay, B.B. (1994) Inhibitors of cytoskeletal function and signal transduction of study bacterial invasion. *Meth. Enz.* **236**:467-476.

[103] Finlay, B.B. and Ruschkowski, S. (1991) Cytoskelelal rearrangements accompanying *Salmonella* entry into epithelial cells. *J.Cell. Sci.* **99**:283-296.

[104] Ed. Ausubel, F.M., Kingston, B.R., Moore, D.D., Seidman, J.G., Smith, J.A and Struhl, K. (1987) *Current Protocols in Molecular Biology*:2.4.1-2.4.2, Wiley Interscience & Green Publishing Associates, New York.

[105] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nuc.Acids Res.* **25**:3389-3402.

[106] Jones, S. and Portnoy, D.A. (1994) Intracellular growth of bacteria. *Meth. Enz.* **236**:463-467.

[107] Beveridge, T.J., Popkin, T.I. and Cole. R.M (1994) Electron microscopy, p.42-71. *In* P. Gerhardt (ed.) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC.

[108] Diem, K. and Lenter, C. (ed.) (1970) Scientific Tables 7th Ed. p280-282. Giegy (Canada) Limited, Montreal, PQ.

[109] White C.A. and Kennedy, J.F. (1986) Orcinol-sulphuric acid assay p.38 *In* Chaplin, MF. and Kennedy J.F. (ed.) Carbohydrate analysis – a practical approach. IRL Press, Washington, DC.

[110] Bryan L.E., Wong, S., Woods, D.E., Dance, D.A.B. and Chaowagol, W. (1994) Passive protection of diabetic rats with antisera specific for polysaccharide protion of the LPS from *Pseudomonas pseudomallei. Can. J. Inf. Dis.* **5**:170-178.

[111] Alms, T.H. and Bass, J.A. (1967) Immunization against *Pseudomonas aeruginosa* I. Induction of protection by an alcohol-precipitated fraction from the slime layer. *J. Inf. Dis.* **117**(3):249-256.

[112] Karkanis Y.D., Zeltner, J.Y., Jackson, J.J. and Carlos, D.J. (1978) A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram negative bacteria. *Anal. Biochem.* **85**:565-601.

[113] Aminoff, D. (1961) Methods for the quantitative estimation of *N*-Acetylneuramic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81**:384-392.

[114] Lieber, M., Smith, B., Szakal, A., Nelson-Rees, W. and Todaro, G. (1976) A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer* **17**:62-70.

[115] Morgan, T.E. (1971) Pulmonary surfactant. *New Engl. J. Med.* **284**:1185-1193.

[116] Makino, K., Shinagawa, H., Amenua, M. and Nakata, A. (1986) Nucleotide sequence of the phoB gene, the positive regulatory gene for the phosphate regulon of *Escherichia coli* K-12. *J. Mol. Biol.* **190**(1):37-44.

[117] Groisman, E.A., Chiao, E., Lipps, C.J. and Heffron, F. (1989) *Salmonella typhimurium* phoP virulence gene is a transcriptional regulator. *Proc. Natl. Acad. Sci.* **18**:7077-7081.

[118] Kim, S.K., Makino, K., Amemura, M, Shinagawa, H., Nakata, A. (1993) Molecular analysis of the phoH gene, belonging to the phosphate regulon in *Escherichia coli. J. Bac.* **175**(5):1316-1324.

[119] Matin, A. and Schultz, J.E. (1991) Molecular and functional characterization of a carbon starvation gene of *Escherichia coli. J. Mol. Biol.* **218**(1):129-140.

[120] Spector, M.P., DiRusso, C.C., Pallen, M.J., Garcia del Portillo F., Dougan, G. and Finlay, B.B. (1999) The medium-/long-chain fatty acyl-CoA dehydrogenase (fadF) gene of *Salmonella typhimurium* is a phase 1 starvation-stress response (SSR) locus. *Microbiol.* **145**(pt1):15-31.

[121] Spector, M.P. (1998) The starvation-stress response (SSR) of *Salmonella. Adv. Microbiol. Physiol.* **40**:233-279.

[122] Liu, J., Duncan, K. and Walsh, C.T. (1989) Nucleotide sequence of a cluster of *Escherichia coli* enterobactin biosynthesis genes: identification of entA and purification of its product 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase. *J Bac.* **171**(2):791-798.

[123] Weinberg, E.D. (1989) Cellular regulation of iron assimilation. *Q. Rev. Biol.* **64**(3):261-290.

[124] Koster, M., van Klompenburg, W., Bitter, W., Leong, J. and Weisbeek, P. (1994) Role for the outer membrane ferric siderophore receptor PupB in signal transduction across the bacterial cell envelope. *EMBO J.* **13**(12):2805-2813.

[125] Van Hove, B., Staudenmaier, H. and Braun, V. (1990) Novel two-component transmembrane transcription control: Regulation of iron dicitrate transport in *Escherichia coli* K-12. *J. Bac.* **172**(12):6749-6758.

[126] Heine, R.P., Elkins, C. Wyrick, P.B. and Sparling, P.F. (1996) Transferrin increases adherence of iron-deprived *Neisseria gonorrhoeae* to human endometrial cells. *Am. J. Obstet. Gynecol.* **174**(2):659-666.

[127] Barnewall. R.E. and Rikihisa, Y. (1994) Abrogation of gamma interferon-induced inhibition of *Ehrlichia chaffeensis* infection in human monocytes with iron-transferrin. *Infect. Immun.* **62**(11):4804-4810.

[128] Badger, J.L, and Kim, K.S. (1998) Environmental growth conditions influence the ability of *Escherichia coli* K1 to invade brain microvascular endothelial cells and confer serum resistance. *Infect. Immun.* **66**(12):5692-5697.

[129] Conte, M.P., Longhi, C., Polidoro, M., Petrone, G., Buonfiglio, V., Di Santo, S., Papi, E., Seganti, L., Visca, P. and Valenti, P. (1996) Iron availability affects entry of *Listeria monocytogenes* into the enterocytelike cell line Caco-2. *Infect. Immun.* **64**(9):3925-3929.

[130] Murata T., Takase, K., Yamato, I., Igarashi, K. and Kakinuma, Y. (1997) Purification and reconstitution of Na+-translocating vacuolar ATPase from *Enterococus hirae. J. Biol. Chem.* **272**(40):24885-24890.