BIOMEDICAL SCIENCES - The role of environmental stress-survival adaptation by Burkholderia pseudomallei in disease emergence.

Summary of Proposed Research Program for Master of Philosophy

Title

The role of environmental stress-survival adaptation *by Burkholderia pseudomallei* in disease emergence.

Abstract

The emergence of a potentially fatal infection in parts of the world where it did not previously exist requires an explanation based on its biology and ecology. Brazil had not recorded any cases of melioidosis prior to 2003 (Rolim *et al.* 2005), but a cluster of cases drew attention to the presence of the disease in the region. Molecular typing indicated a possible link with an Australian outbreak strain. Subsequent genotyping by whole genome sequencing indicates that the Brazil outbreak strain belongs to a distinct geographic clade, separate from the main Australian and Southeast Asian clades of *B. pseudomallei*. Both these strains have been fully sequenced by the Naval Medical Research Center Annex in Rockville, MD; a pathogenomics group closely associated with the J. Craig Venter Institute (formerly known as TIGR). We are now ready to use advanced bioinformatic tools to systematically interrogate the two *B. pseudomallei* outbreak strain genomes. The proposed project will use this data in an integrated systems biology analysis of the two outbreak strains in order to understand how they differ from non-outbreak strains of *B. pseudomallei* that cause sporadic single-case infection and environmental isolates not known to cause any human or animal disease. This project will therefore test the hypothesis that disease emergence in the case of melioidosis is a function of environmental survival. The project will develop the concept of *B. pseudomallei* virulence as an adaptive response to a novel environment. The project will use a combination of whole bacterial genome sequence, molecular and cell biology methods and applied bioinformatic techniques to understand the basis of biological threat agent adaptation to physico-chemical stress that may induce sublethal damage, a viable but non-culturable state and reversible morphological change. The project will take whole genome sequence data as a starting point and examine survival using reverse transcription polymerase chain reaction (RT-PCR), gene expression microarrays, flow cytometry and advanced microscopic techniques.

Objectives

The hypothesis is that disease emergence in the case of melioidosis is a function of environmental survival pressures on *B. pseudomallei*.

The main intent of this project is to understand the contribution of bacterial survival and environmental stress adaptation to disease emergence in the specific instance of *B. pseudomallei* and melioidosis, using molecular biology insights gained from whole genome sequencing of the *B. pseudomallei* to understand the relationship between environmental adaptation and survival under conditions of physico-chemical stress.

Consequently the project aims to understand the molecular basis of *B. pseudomallei* cellular adaptation to adverse environmental conditions including sublethal damage, the viable but non-culturable (VBNC) state and reversible morphological change. Hence the objectives of the project are to:

a. Identify genes that are up- or down-regulated in response to stress conditions.
b. Understand the connection between gene expression and structural changes in B. pseudomallei that are brought about by adaption to stress conditions.
c. Identify survival mechanisms that are specific to *B. pseudomallei by* comparing the genomes of *B. thailandensis* and *B. mallei* with the *B. pseudomallei*.
d. Identify common survival mechanisms that may apply to a range of organisms by comparing the genomes of *B. thailandensis* and *B. mallei* with the *B. pseudomallei*.
e. Examine a range of environmental and clinical *B. pseudomallei* isolates from the *Burkholderia* Culture Collection to evaluate the prevalence of these survival mechanisms.

Background

Melioidosis is a potentially fatal bacterial infection caused by the Gram negative bacillus, *Burkholderia pseudomallei*. The first description of disease came from Burma in 1911 (Whitmore 1912) and has since been reported in a large number of countries. However due to the difficulties of diagnosis, it is likely that disease occurs more frequently than is reported (Dance 2000).

Geographical distribution

The disease is endemic throughout the world's tropical zone; the majority of reported cases occur in south-east Asia, though melioidosis has been reported from many countries including in South America, and the Middle East (Dance 1991; Dance 2000). In Australia disease occurs in the tropical north of Western Australia, the Northern Territory and Queensland, and sporadic disease in animals and occasionally humans has occurred as far south as Toodyay in Western Australia. Most of the recognised disease occurs in Thailand, but it is likely this is due to greater recognition of disease or due to an increase in disease related to changes in human-environment interaction (Dance 2000). *B. pseudomallei* is known to be capable of prolonged persistence in the environment, particularly in soil (Thomas and Forbes-Faulkner 1981) and has been isolated from many environmental samples (Smith *et al.* 1995). The environment, particularly the tropical rhizosphere, is thus the likely source of melioidosis.

Disease

The disease can take many forms, and hence is difficult to diagnose without laboratory confirmation. Melioidosis can take the form of rapidly progressing septicaemia, pneumonia, a localised soft tissue infection or a sub-clinical infection that can later become an evident infection (Inglis and Sagripanti 2006; Cheng and Currie 2005). Treatment can be difficult due to rapid progression or antibiotic resistance, and is usually prolonged. Relapse can occur even with prolonged treatment with appropriate antibiotics. The route of acquisition is not fully understood, though it is thought possible via inhalation, ingestion or percutaneous inoculation. Verifiable, point source outbreaks are extremely unusual in humans, the main manifestation of acute, septicaemic disease being a peak of culture-confirmed cases during the tropical rainy season, especially following heavy monsoonal rainfall (Currie and Jacups 2003). Though indigenous Australians are at high risk of infection due to recognised underlying disease risk factors such as diabetes and chronic renal failure, sporadic infections occur in tourists, shire and mine workers and recreational gardeners, some of who have no identifiable explanatory risk factors (Inglis and Sagripanti 2006; Cheng and Currie 2005). Person-to-person spread is not a feature of melioidosis.

Occasional outbreaks of disease have occurred, most notably in the Kimberley region in 1997 (Inglis *et al.* 1998) and in Brazil in 2003 (Rolim *et al.* 2005). The emergence of melioidosis in Brazil was so abrupt that the initial outbreak investigation team considered bioterrorism as a possible explanation (TJJ Inglis, personal communication). More plausible explanations now include intrusion events precipitated by livestock import or crop plant introduction. Outbreaks of melioidosis therefore present a rare opportunity to investigate the specific contributory factors whose convergence led to a change in melioidosis epidemiology. These factors are a combination of host, pathogen and mediating environment. In the case of the West Kimberley outbreak, melioidosis occurred in a location where the disease was already well recognised, in subjects with identifiable co-morbidities. The Brazil outbreak, on the other hand, occurred where the disease had not been diagnosed previously in a group of otherwise healthy children with no recognisable risk factors. The Brazil outbreak strain therefore appeared to be more virulent and thus less well adapted to a human host, as would be consistent with a recent disease incursion.

Environmental survival

The environmental survival mechanisms used by *B. pseudomallei* to persist in the environment have not been as thoroughly investigated as potential virulence factors. However *B. pseudomallei*, as well as other environmental *Burkholderia* species appear to survive in seemingly inhospitable conditions for extended periods of time, such as in soil in the Kimberley region over the dry season. The most notable experiment into survival showed *B. pseudomallei* survived in sterile distilled water without nutrients for three years (Wuthiekanun, Smith, and White 1995), and cells from these continuing experiments remain culturable (Inglis and Sagripanti 2006). Other early work was done examining the survival potential of *B. pseudomallei* in tap water (Miller *et al.* 1948) and in soil (Thomas and Forbes-Faulkner 1981; Tong *et al.* 1996). Tong *et al.* also examined survival at varying temperature and pH values. Some work in more recent years on survival has been conducted on survival of *B. pseudomallei* and *B. cepacia* in amoeba (Inglis *et al.* 2000; Inglis *et al.* 2003; Lamothe, Thyssen, and Valvano 2004) and chlorine tolerance of *B. pseudomallei* (Rose *et al.* 2005; Howard and Inglis 2005). At the molecular level, the *rpoE* operon and *rpoS* gene have been implicated in survival and a stress response (Korbsrisate 2005; Subsin *et al.* 2003; Thongboonkerd *et al.* 2007; Vanaporn *et al.* 2008). The *rpoE* operon exerts control over oxidative and osmotic stress response proteins, chaperones,

transcriptional/translational regulators and proteins involved in cell wall synthesis (Thongboonkerd *et al.* 2007; Korbsrisate 2005), and regulates the heat-stress response through the function of *rpoH* (Vanaporn *et al.* 2008); the *rpoS* gene in Gram-negative bacteria (such as *Enterobacteriaceae* and *Pseudomonadaceae*) activates expression of genes in response to acid and heat shock, UV, osmotic and oxidative stress and carbon starvation (Subsin *et al.* 2003) and controls the activities of catalases I and II (Jangiam, Loprasert, and Tungpradabkul 2008). A recent experiment on the survival of *B. pseudomallei* in water showed an increase in the expression of a gene encoding for a putative membrane protein (Moore, Tuanyok, and Woods 2008).It is likely that a very large number of genes contribute to the ability of *B. pseudomallei* to survive in the environment. The genes discussed above give some clues to survival mechanisms used and can be used as a starting point for investigation.

Survival experiments

The Melioidosis Research group based at PathWest lodged the Kimberley outbreak strain with the National Collection of Type Cultures in 2000 as NCTC 13177 and this strain has been used extensively in our laboratory studies as an Australian reference strain. NCTC 13177 has been the subject of intensive environmental survival studies under physico-chemical conditions that designed to simulate specific circumstances of the Kimberley environment in which it was originally isolated (unpublished data). A standard survival method has been developed under the guidance of the Edgewood ChemBio Center. This has been used to confirm our original observations of tolerance and survival despite exposure to 100ppm chlorine (Howard and Inglis 2003). *B. pseudomallei* has been shown by us to undergo stress-induced morphological adaptation including formation of coccoid, Gram positive forms, and elongated spiral bacilli (Robertson *et al.* 2007). A metabolic reserve enabling survival during environmental stress appears to lie in the secondary metabolism of *B. pseudomallei*, which forms a substantial carbon store in granules of polyhydroxyalkanoates during late log phase. We identified the structural gene responsible for polyhydroxybutyrate synthesis (*phaC*) in *B. pseudomallei* and have begun to explore the potential role of the PHB operon as a contributor to bacterial survival and virulence (Merritt *et al.* 2006); GenBank # DQ083762).

Genomics

During early 2007 the two outbreak strains NCTC 13177 and Bcc 215 (Brazil outbreak strain), a sporadic infection strain DM98 and two near-neighbour *Burkholderia* spp. (*Burkholderia thailandensis* Bt4 and *Burkholderia ubonensis* Bu, both recovered from the Kimberley region) were sequenced to a whole genome level by our collaborators in Rockville. Several more strains of *B. pseudomallei* and a near neighbour *Burkholderia* spp. are going to be sequenced in the next year. The first series of fully sequenced Burkholderia genomes from the WABCC have gone through preliminary annotation and can be viewed on GenBank (NZ_ABBQ00000000, NZ_ABBR00000000, NZ_ABBI00000000, NZ_ABBH00000000, NZ_ABBE00000000 respectively). More recently, the Comparative Microbial Genomics group at the Center for Biological Sequence Analysis (Technical University of Denmark) have created and shared with us a genomic atlas of 34 *B. pseudomallei* and near neighbour *Burkholderia* strains; a resource that will be helpful to this project. The preliminary gnomic tree constructed from this genomic atlas clarified the relatedness of the Kimberley and Brazil outbreak strains. Initial molecular typing had shown the two strains to be closely related, but the whole genome comparison placed the two strains much further apart on the genomic tree.

The implications of these results include a likely revision of previously assumptions regarding the geographic origins of melioidosis and glanders. Included in the genomic atlas are some isolates of the near neighbour species *B. thailandensis*, which can survive in environment but does not cause disease, and *B. mallei*, which cannot survive outside an animal host and can cause human disease. This means that the areas of the genome that contain genetic differences responsible for the differing environmental survival and virulence capabilities can be easily located using the genomic atlas, then expanded to gene sequence or even base pair level without further in vitro experiment. This strategy is being used by researchers to elucidate the evolutionary relationship between *B. mallei* (Moore *et al.* 2004; Nierman, DeShazer, *et al*. 2004; Ong *et al.* 2004) and *B. thailandensis*(Kim, Schell, *et al.* 2005; Ong *et al*. 2004). Ong *et. al.* (2004) found that there was less than 3% genomic variation between *B. pseudomallei* isolates examined, and that *B. mallei* and *B. thailandensis* diverged from *B. pseudomallei* and each other via distinct mechanisms. The major distinction between *B. thailandensis*, and *B. pseudomallei* and *B. mallei* is the ability to metabolise arabinose (Brett, DeShazer, and Woods 1998), and this is discussed by Moore *et al.* (2004).

Significance

Outbreaks of melioidosis present a rare opportunity to investigate the specific contributory factors whose convergence led to a change in melioidosis epidemiology. These factors are a combination of host, pathogen and mediating environment. Access to the Western Australian and Brazil outbreak strain cultures and genomes is unique and this work will be the first to examine these strains in great detail from the genome to the environment. The fully sequenced genomes will allow the use of bioinformatics and molecular techniques to elucidate the pathogen factors that contributed to the outbreaks.

While many groups have started to use bioinformatics techniques to increase their understanding of *B. pseudomallei*, most have been focussing on the virulence genes. This project takes a wider view of the emergence of melioidosis, and examines *B. pseudomallei* as part of a complex and interconnected natural environment, rather than purely in a laboratory or an animal model. By examining *B. pseudomallei* environmental survival from the level of genome sequence, to gene expression and to the phenotypic level, a more complete view of any contributory factors can be obtained. A greater understanding of how the organism can survive adverse environmental conditions should provide some insight into disease issues such as the process of disease emergence, decontamination and the potential for disease spread by contaminated soil, water or livestock.

Research methods

a. Applied bioinformatics. The whole bacterial genomes of *B. pseudomallei* strains used in preliminary survival experiments will be used as a template for gene expression microarray design and use. Microarrays are glass slides with a large number of attached oligonucleotides. The oligonucleotides (probes) are selected to bind to sections of DNA (targets) from genes of interest. To create the targets, mRNA (messenger RNA, the transcript used to make proteins) is extracted from the cells and converted to DNA. This allows us to measure which genes are being transcribed at a given time, and with controls, changes in transcription can also be measured (see (Dharmadi and Gonzalez 2004) for a comprehensive overview). Microarrays will be used to identify genes up- and down-regulated when cells are stressed. Subjecting populations of bacteria to adverse conditions such as starvation, temperature extremes, acid conditions and the presence of disinfectants can induce stress. The population of cells that survive in adverse conditions are known to have adjusted gene expression to survive (Korbsrisate 2005; Subsin *et al.* 2003; Vanaporn *et al.* 2008). The adaptive functions we will focus on include carbon storage, SOS DNA repair repression and induction functions (Michel 2005), cell wall and membrane repair (Moore, Tuanyok, and Woods 2008) and regulatory genes such as *rpoS* (Subsin *et al.* 2003) and *rpoE* (Korbsrisate 2005). These functions have been shown to be important in stress response in *Burkholderia* or other bacterial species and are likely to be involved in the *B. pseudomallei* stress response. Confirmatory studies will be conducted with gene expression PCR probes designed to amplify specific structural or regulatory genes of interest.

b. Applied cell biology. The gene expression arrays and PCR probes will then be used in conjunction with flow cytometry and *in vivo* electron microscopy to understand the connection between molecular and structural changes brought about by adaptation to physical and stressors such as acidic pH, high osmotic pressure and temperature extremes. Flow cytometry allows examination of individual bacterial cells and enables measurement of various properties of each cell examined such as, cell sizeshape, membrane permeability or metabolic activity. Scanning electron microscopy shows the surface of whole cells with depth of field, so the cell morphology can be examined in detail. Transmission electron microscopy can quickly give an image of the cell without depth of field, or can be used to show cellular contents by slicing through the cell. These techniques should show any morphological changes. Fluorescent *in situ* hybridisation (FISH) will be used to demonstrate key gene functions by surviving bacteria exposed to stress on a solid surface. This is done using similar methods to microarray techniques. Rather than using an anchored probe the probe is instead free in solution and able to penetrate intact cells and bind to its complementary mRNA molecule. The fluorescence induced on binding is observed optically on a microscope, thereby enabling identification of expression of a given gene or activity of a gene product. . Field work in *B. pseudomallei* contaminated locations in the Kimberley and Sri Lanka will be conducted to test the plausibility of candidate gene(s) as survival-emergence determinants *in campo* using our field-deployable laboratory. This will involve the use of field-portable molecular biology and improved sampling methods.

c. Integration of molecular and cellular methods. The series of molecular and cell biology methods with which the stress-survival-emergence response has been identified will be fine-tuned by metagenomic analysis of Burkholderia genomes conducted in a complementary parallel project (the metagenomic analysis is being conducted by another PhD student). Comparisons with *B. thailandensis* and *B. mallei* genomes using BLAST will be used to identify *B. pseudomallei* specific survival mechanisms. BLAST compares the nucleotide sequences and identifies matching sequence. By comparing *B. pseudomallei* with other biothreat agent genomes (including *B. anthracis* and *Coxiella burnettii*), common bacterial survival strategies or pathways can be identified. The predictive value of this analytical process will be evaluated by a targeted analysis of *B. pseudomallei* isolates from the WA Burkholderia Culture Collection to identify specific strains with high potential for disease emergence in the event of increased cyclonic activity or other macro-environmental determinants.

Ethical issues

No research will be performed that uses humans or animal subjects. Bacterial strains isolated from humans have outline context information in the BCC historical archive. Our analyses will respect the confidentiality of all subjects from whom BCC isolates have been obtained. No genetic recombination experiments will be undertaken.

Facilities and resources

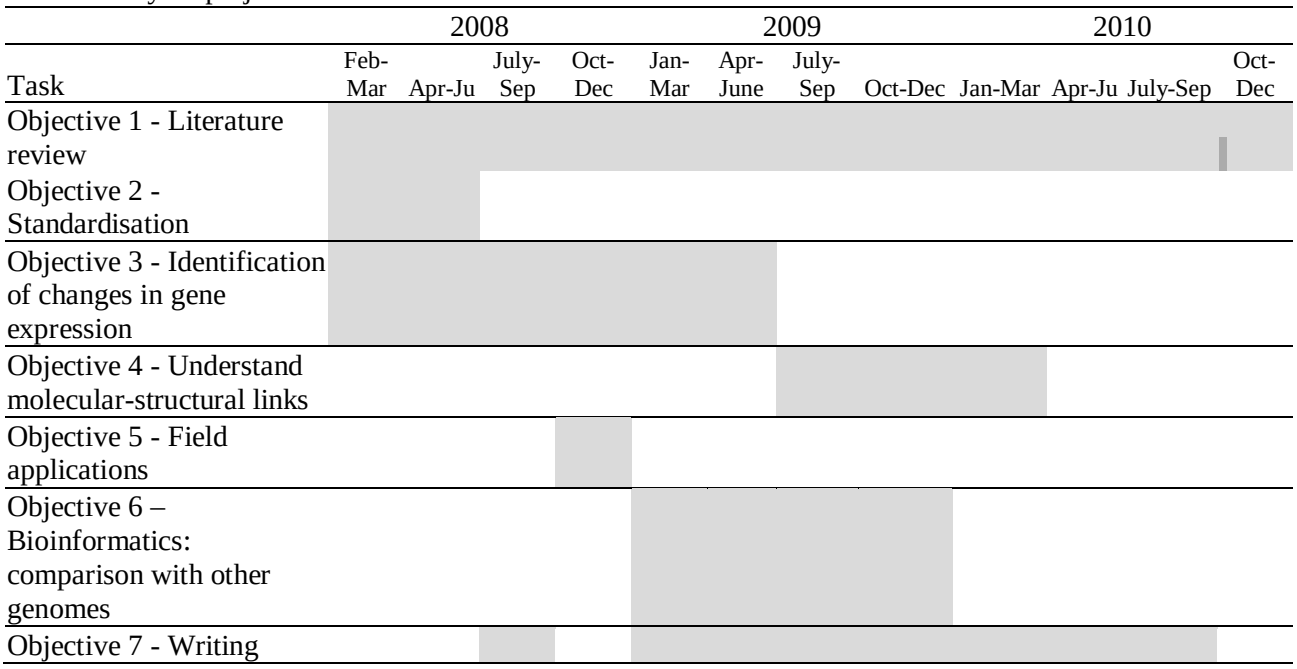
The Division of Microbiology and Infectious Diseases, PathWest Laboratory Medicine WA will provide most baseline facilities and resources required for this project, as the majority of experimental time will be spent at there. Office space, use of a computer, telephone, fax, photocopier, stationary supplies and statistical software are all provided. Laboratory space, access to basic laboratory equipment, reagents and PCR equipment are provided. Access to microarray facilities is anticipated either at Curtin University (Biosecurity CRC) or PathWest. An interim arrangement is under discussion with the Victorian AgroBiosciences Centre at La Trobe University, VIC with the support of Agilent Australia Pty. Electron microscope use will be through the CMCA.

Data Storage

The data storage provisions are outlined in the attached Research Data Management Plan and meet the Curtin University Research Data and Primary Materials Policy.

Time Line

A proposed time line is attached outlining the schedule of laboratory work and writing activity for the entire three year project.



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