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Attenuation of a select agent-excluded *Burkholderia pseudomallei* capsule mutant in hamsters

Maria G. Gutierrez [a], Jonathan M. Warawa [a,b,*]

[a] Department of Microbiology and Immunology, University of Louisville, Louisville, KY 40202, United States
[b] Center for Predictive Medicine, University of Louisville, Louisville, KY 40202, United States

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ABSTRACT

Burkholderia pseudomallei is a Tier 1 select agent and potential bioweapon. Given it is potential to cause a lethal respiratory disease, research with fully virulent *B. pseudomallei* is conducted in Biosafety Level 3 (BSL-3) laboratory spaces. The logistical, financial, and administrative burden of Tier 1 select agent BSL-3 research has created an interest in mitigating such burdens through the use of either attenuated *B. pseudomallei* strains at BSL-2, or research with surrogate species, such as *Burkholderia thailandensis*. Previously, attenuated *B. pseudomallei* auxotroph mutants (*asd* and *purM*) have been approved for exclusion from select agent requirements, allowing for *in vitro* studies to be conducted at BSL-2. Acapsular *B. pseudomallei* mutants are known to be strongly attenuated in a variety of animal models, and yet acapsular *B. pseudomallei* mutants do not require nutritional supplementation, and can be studied within cultured macrophages, performing phenotypically similarly to parent strains. We demonstrate that the loss of a 30.8 kb region of the *wcb* capsule operon allows for a dramatic >4.46 log attenuation in a hamster intraperitoneal infection model, and report that this strain, JW270, has met criteria for exclusion from select agent requirements.

1. Introduction

Burkholderia pseudomallei is a gram-negative bacterial pathogen which is classified as a Tier 1 select agent. *B. pseudomallei* is responsible for the disease melioidosis, and is endemic to Southeast Asia and northern Australia with increasing prevalence worldwide (Cheng and Currie, 2005; Inglis et al., 2006; Wiersinga et al., 2006; Schweizer et al., 2014). The bacterium is found in moist tropical environments from which it opportunistically infects a variety of plant, vertebrate, and invertebrate hosts. The most prevalent route of infection in humans is thought to be percutaneous inoculation, however additional common routes of infection include ingestion or inhalation. In all cases, poor disease outcome is associated with a highly lethal septicemic disease progression.

Studies with fully virulent *B. pseudomallei* require use of a BSL-3 laboratory space to mitigate the risk of respiratory spread. Further, *B. pseudomallei* is a select agent organism requiring regulatory oversight of research programs, involving numerous federal agencies. Finally, in the United States, *B. pseudomallei* has recently been identified as a Tier 1 select agent which requires further scrutiny of research programs at the institutional level in the form of Personnel Reliability Programs. With the ever growing administrative burden of conducting research with fully virulent *B. pseudomallei* research, many laboratories have sought out alternative strategies involving use of attenuated strains or surrogate species. *B. thailandensis* has been widely accepted as a genetically closely-related surrogate for a variety of cell culture and animal model studies, as it does share most of the understood virulence systems found in *B. pseudomallei*, with the exception of capsular polysaccharide (Sim et al., 2010). Alternatively, use of attenuated auxotrophs of *B. pseudomallei* allows for investigations to be conducted using isogenic strains to study a variety of *B. pseudomallei*-specific genetic systems. Research involving use of auxotrophs may be limited to *in vitro* studies where nutritional supplementation can be provided to overcome otherwise lethal mutations. At present, two such auxotrophs in aspartate-B-semialdehyde dehydrogenase (*asd*) and purine biosynthesis (*purM*) genes have been demonstrated to result in dramatically attenuated strains that have secured approvals for exclusion from the select agent requirements (Propst et al., 2010; Norris et al., 2011).

We have previously generated a 30.8 kb capsular polysaccharide cluster mutant, strain JW270, which we found is capable of being

* Corresponding author at: Department of Microbiology and Immunology, University of Louisville, 505 S. Hancock St., CTRB 619, Louisville, KY 40202, United States. Fax: +1 502 852 5468.
E-mail address: jonathan.warawa@louisville.edu (J.M. Warawa).

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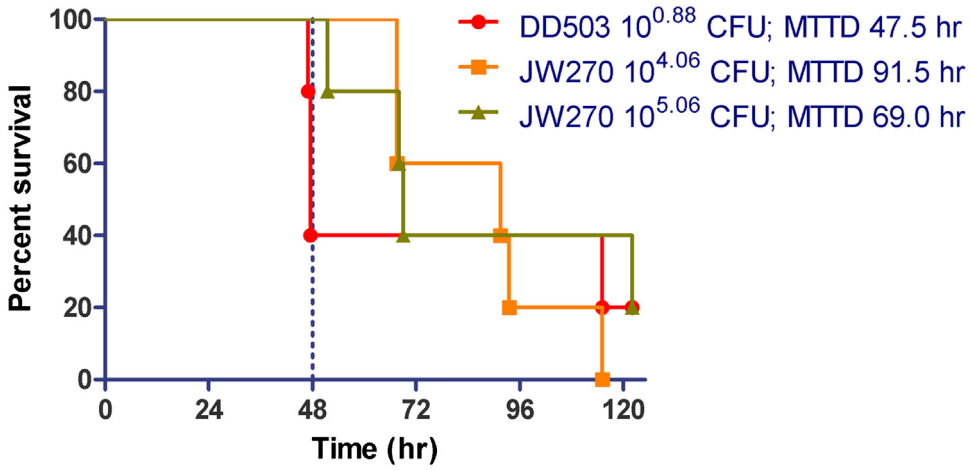

Fig. 1. Survival analysis of JW270 in hamsters. *B. pseudomallei* strains DD503 (parent) and JW270 (capsule mutant) were used to infect hamsters ($n = 5$/group) by the intraperitoneal route with the doses indicated. Hamsters were euthanized at moribund endpoints from health checks conducted four times per day. The median time to death (MTTD) was calculated in GraphPad Prism, as was Log-rank survival analysis.

internalized into cultured macrophages and proliferating in this intracellular niche at levels similar to the parent strain (Warawa et al., 2009). Use of capsule mutants to study the intracellular lifestyle of *B. pseudomallei* therefore represents an attractive alternative to use of surrogate species or auxotrophs which may not be nutritionally rescued within the intracellular space. Single gene capsule mutants have been previously demonstrated to be severely attenuated (>5 log) in systemic disease models within both murine and hamster infection models (Reckseidler et al., 2001; Atkins et al., 2002; Reckseidler-Zenteno et al., 2005). Thus, capsule biosynthetic cluster mutants offer an attractive option as attenuated strains that might be excluded from select agent requirements, and yet facilitate intracellular lifestyle studies within cell culture model systems.

Two key considerations for an attenuated strain to be considered for removal from select agent requirements include: (1) "level of difficulty in engineering the attenuated strain to restore wild-type virulence" and (2) "quantitative measures demonstrating a change in virulence in an appropriate animal or plant model." (http://www.selectagents.gov/resources/Exclusion_Guidance_Document_01292015.pdf). We propose that the removal of 30.8 kb of capsule polysaccharide genes meets this first requirement, and in these studies we further address the second criteria by demonstrating dramatic attenuation of acapsular *B. pseudomallei* strain JW270 in the well described hamster model.

2. Materials and methods

2.1. Bacterial strains and culture

B. pseudomallei strains DD503 (Moore et al., 1999) and JW270 (Warawa et al., 2009) were routinely cultured in Lennox Broth (LB) at 37 °C. For inoculum preparation, *B. pseudomallei* strains were subcultured 1:25 in dialyzed and chelated Trypticase Soy Broth (TSBDC) supplemented with 50 μM monosodium glutamate from overnight LB cultures and grown for 3 h at 37 °C.

2.2. Ethics statement

All animal studies were conducted under Biosafety Level 3 conditions using six to eight week old female Golden Syrian Hamsters (Crl:LVG (SYR), Charles River). These studies were approved by the University of Louisville Institutional Animal Care and Use Committee (Protocol number 13053) in agreement with NIH guidelines and the "Guide for the Care and Use of Laboratory Animals" (NRC).

2.3. Virulence assessment of acapsular JW270 strain

Hamsters were implanted with an identification/temperature transponder (Biomedic Data Systems) subcutaneously at the scruff of the neck upon arrival, under isoflurane anesthesia. Hamsters were infected with 100 μl of bacterial/PBS suspensions by intraperitoneal inoculation, as described (Brett et al., 1997). At 24 h post-infection, health checks were conducted four times per day to monitor fitness and temperature. Animals were euthanized when righting reflex was absent or at study completion at day 5. Following euthanasia by carbon dioxide asphyxia, animals were exsanguinated by cardiac draw, and tissues were collected for bacterial enumeration (lung, liver, and spleen) as described (Lawrenz et al., 2014).

2.4. Statistical analysis

Two-way ANOVA followed by the Bonferroni post-test and Log-rank survival analysis were conducted using GraphPad Prism.

3. Results

3.1. Acapsular JW270 is attenuated in the hamster model

We used the well-established intraperitoneal hamster 48 h infection model to quantify the degree of attenuation of an acapsular JW270 strain. The parental strain DD503 was used as a control, and a group challenged with $10^{0.88}$ CFU of DD503 experienced 60% mortality at the 48 h endpoint (Fig. 1), which is consistent with the previously reported 48 h LD_{50} of this strain of $10^{0.60}$ CFU (Warawa and Woods, 2005). By 48 h, challenge with neither $10^{4.06}$ nor $10^{5.06}$ CFU of the acapsular JW270 strain caused mortality, suggesting that this strain is >4.46 log attenuated over the reported LD_{50} of DD503.

We continued to monitor disease progression beyond the established 48 h endpoint and found that JW270-infected hamsters did succumb to disease at delayed time points relative to the parental control group (Fig. 1). While the median time to death (MTTD) was delayed in JW270-infected hamsters, this delay was not significant by Log-rank test. Monitoring beyond the established 48 h endpoint consistently demonstrated a delay in the MTTD of a $10^{5.06}$ CFU challenge of JW270 relative to $10^{0.88}$ CFU challenge with the parental DD503 strain.