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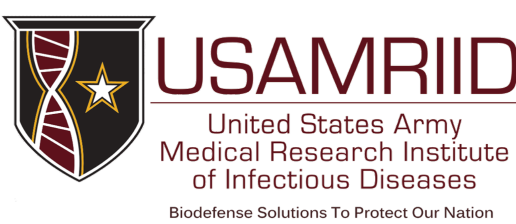

Spontaneous mutations within O-antigen synthesis genes contribute to phase variation in *Francisella tularensis*

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Introduction

As the causative agent of tularemia, *Francisella tularensis* is a highly virulent intracellular pathogen that is found throughout the northern hemisphere. *F. tularensis* can replicate intracellularly in a broad range of cellular hosts including single-celled amoeba and arthropods, with outbreaks most often associated with small mammals. While a small number of human cases occur annually, *F. tularensis* poses a significant threat to human health and is classified as a Tier 1 select agent due to a low infectious dose, high morbidity, and ease of aerosolized inoculation. With this in mind, there has been a renewed effort to identify new medical countermeasures to combat this threat. A Live Vaccine Strain (LVS) exists to prevent tularemia, but an unknown ancestral past and poorly understood basis of attenuation prevents wide-spread use and FDA licensure of this vaccine. Furthermore, spontaneous phase variation of colony morphology between "blue" or "gray" states is known to adversely affect immunization when using LVS, as gray variants greatly decrease the vaccine efficacy. In an effort to understand phase variation in *F. tularensis*, we identified an easily traceable natural subpopulation of gray variants that features a distinct colony morphology that appears in aged cultures (**Figure 1**).

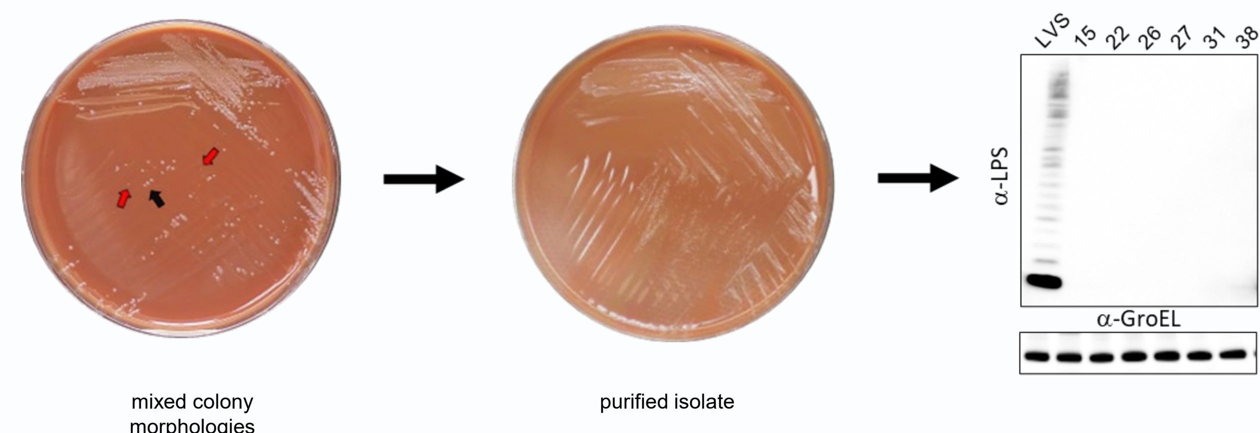


Figure 1: Identification of gray variants based on colony morphology and western blotting. LVS cultured in CDM was streaked on chocolate agar and grown for 3 days at 37°C. Representative images are shown. Black arrow indicates large colony morphology, similar to wild type while red arrows indicate the small colony morphology observed in gray variants. Small colonies were streak purified and confirmed to be gray variant by western blot analysis using an antibody specific for *F. tularensis* O-antigen.

Genomic Analysis

Previous work with gray variants selected on colony morphology demonstrated they were unable to be "reset" to a blue form upon subculturing, which led us to hypothesize genetic differences may be responsible for the observed variation (1). We independently isolated gray variants and performed whole genome sequencing which revealed lesions within the *wbtJ* gene, a formyltransferase involved in O-antigen synthesis. These lesions included SNPs as well as insertions and deletions and a nonsense mutation (**Table 1**).

STRAIN	POSITION	TYPE	REF	ALT	NT POS	AA POS	EFFECT	MUTATION
LVS Isolate #15	1315079	snp	C	T	459/726	153/241	nonsense	Gln153*
LVS Isolate #22	1314645	snp	C	A	23/726	8/241	missense	Thr8Lys
LVS Isolate #26	1314764	deletion	AGTGAGATTAAGCCAATAG	A	143/726	48/241	frameshift	Ser48fs
LVS Isolate #27	1315196	deletion	ATGTG	A	577/726	193/241	frameshift	Cys193fs
LVS Isolate #31	1314969	snp	G	A	347/726	116/241	missense	Gly116Glu
LVS Isolate #38	1314834	snp	C	T	212/726	71/241	missense	Ser71Phe

Detection of gray to blue variation *in vitro*

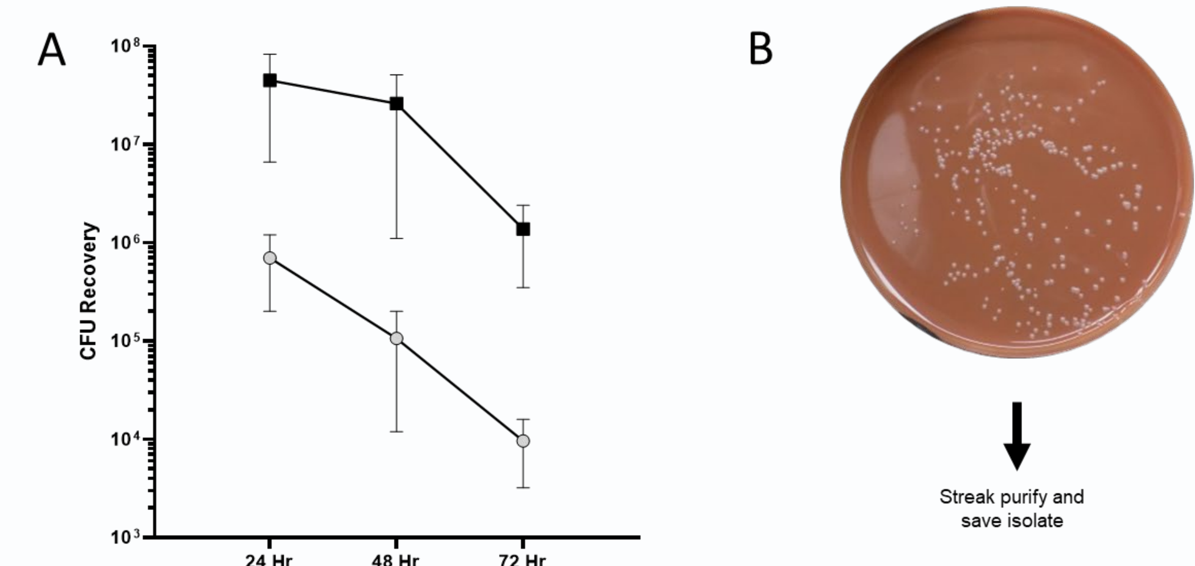
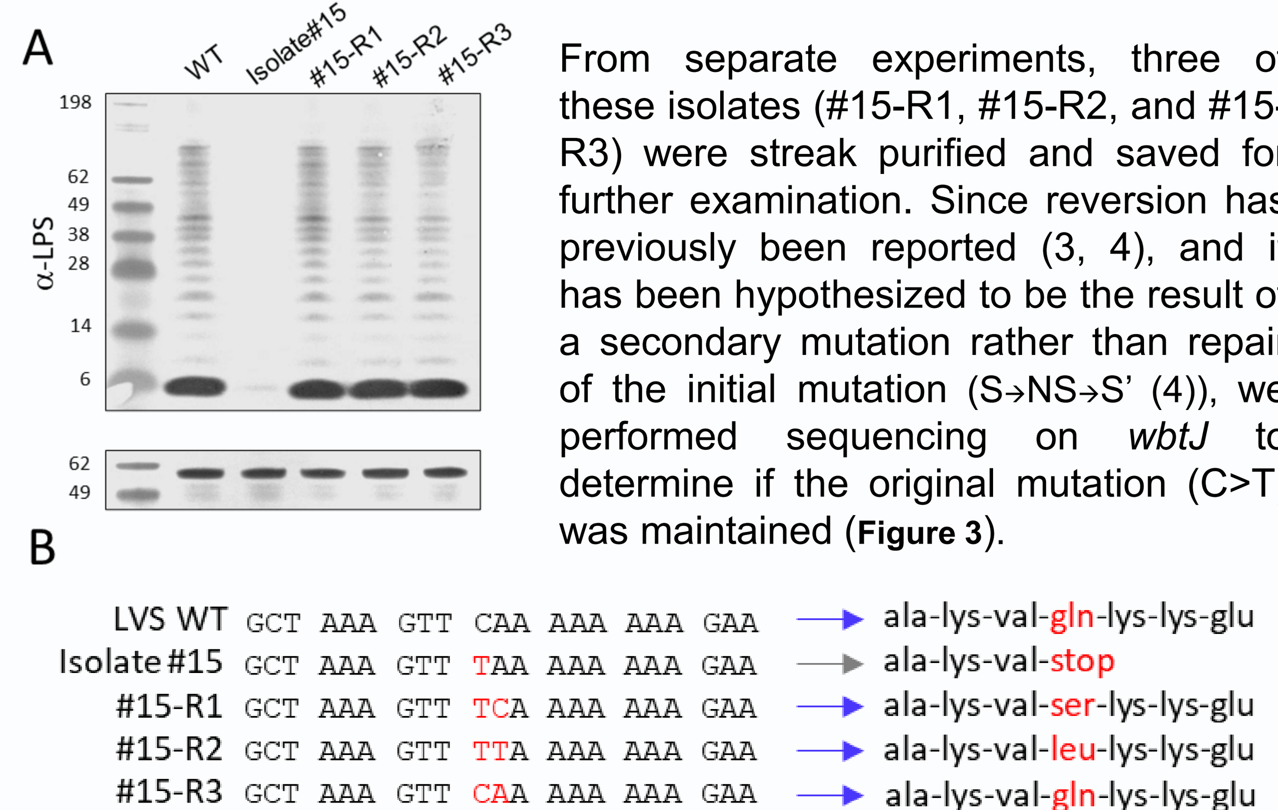


Figure 2: Identification of gray variants based on colony morphology and western blotting. (**A**) J774A.1 macrophages were infected with Isolate #15 and assayed over the course of 3 days. Error bars represent the standard error of the mean. (**B**) A heterogenous mixture of big and small colonies was observed in samples plated from macrophage assays with Isolate #15 each time an experiment was conducted. Colonies that appeared larger than the parent were streak purified and analyzed by western analysis and sequencing.

It was previously demonstrated that gray variants of LVS with alterations to the O-Ag had a survival defect in mouse macrophages (2). With this in mind, we sought to first assess the ability of Isolate #15, a gray variant harboring a nonsense mutation in *wbtJ*, to replicate within J774A.1 macrophages. Isolate #15 was unable to replicate as robustly as the parental wild type at 24 h post infection. To ensure the observed fitness defect was not due to delayed replication, we also tested 24 h and 48 h post infection. An increase in CFU recovered was not observed for Isolate #15 at any timepoint (**Figure 2**). While performing experiments with infected macrophages, we observed a low-level variation in the colony morphology in the natural biofilm forming variants (<20 colonies in total per experiment), particularly at 24 h post infection and beyond. Isolated colonies were selected, and streak purified. Upon re-streaking, the colony morphology appeared to resemble the LVS wild-type morphology as the colonies were larger and less opaque than isolate #15 used in the assay.



From separate experiments, three of these isolates (#15-R1, #15-R2, and #15-R3) were streak purified and saved for further examination. Since reversion has previously been reported (3, 4), and it has been hypothesized to be the result of a secondary mutation rather than repair of the initial mutation (S→NS→S' (4)), we performed sequencing on *wbtJ* to determine if the original mutation (C>T) was maintained (**Figure 3**).

Figure 3: Natural reversion events within the *wbtJ* gene can be detected in macrophage passaged Isolate #15 samples. (**A**) Western analysis and (**B**) **s**equencing was performed on the *wbtJ* gene to determine the allele of the reverted isolate. Red indicates a substituted nucleotide compared to the LVS wild-type sequence for this gene.

Reversion of gray to blue occurs *in vivo*

Given that the natural LVS variant displayed a fitness defect in macrophages but some level of replication still occurred, we sought to determine if this variant (LVS Isolate #15) was virulent in mice via intranasal challenge. Mice challenged with Isolate #15 did not display signs of illness, and we could only determine that the LD_{50} was >45,000 CFU (**Figure 4**).

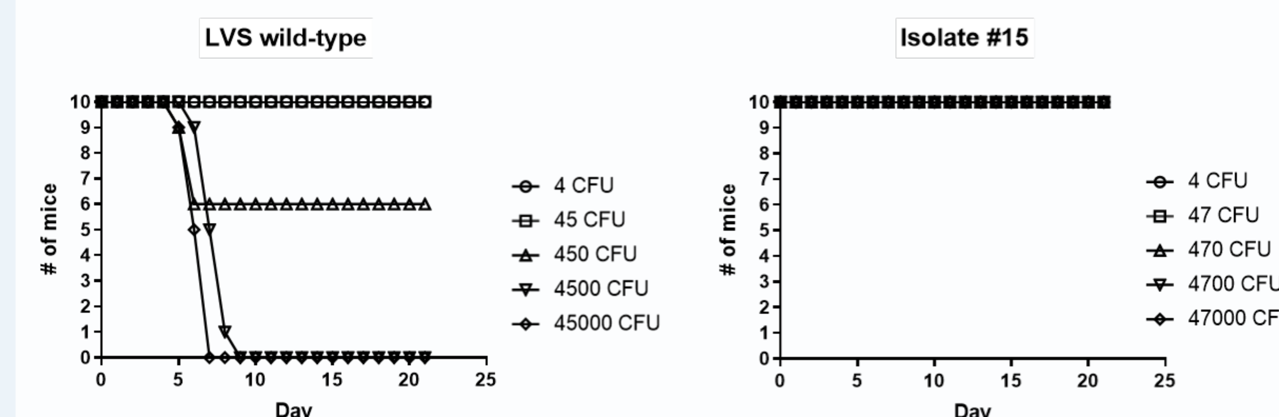


Figure 4: Gray variant isolate #15 is completely attenuated in a pneumonic tularemia mouse model. Mice were challenged intranasally with doses ranging from 1 to 10^5 CFU for the LVS wild-type (parent) and Isolate #15. Displayed are the survival curves for groups of 10 mice following the mice for 21 days post-challenge.

At 21 days post-challenge, spleens were harvested from survivors of isolate #15 to check for sterility or bacterial burden. Only the highest challenge group contained detectable levels of bacteria (~174 CFU/spleen). Surprisingly, all colonies displayed a morphology similar to wild type. At random, 30 colonies encompassing all 10 spleens were re-streaked and sequenced to analyze the *wbtJ* gene. To our surprise, all re-streaked isolates contained a "repaired" version of *wbtJ* as was observed for the reverted isolated from macrophages in earlier studies with reversion to glutamine or leucine at residue 153 detected in these samples. Additionally, normal O-Ag banding was observed when western blot analysis was performed. Collectively, these results suggest that reversion of biofilm forming variants occurs *in vivo* (**Figure 5**).

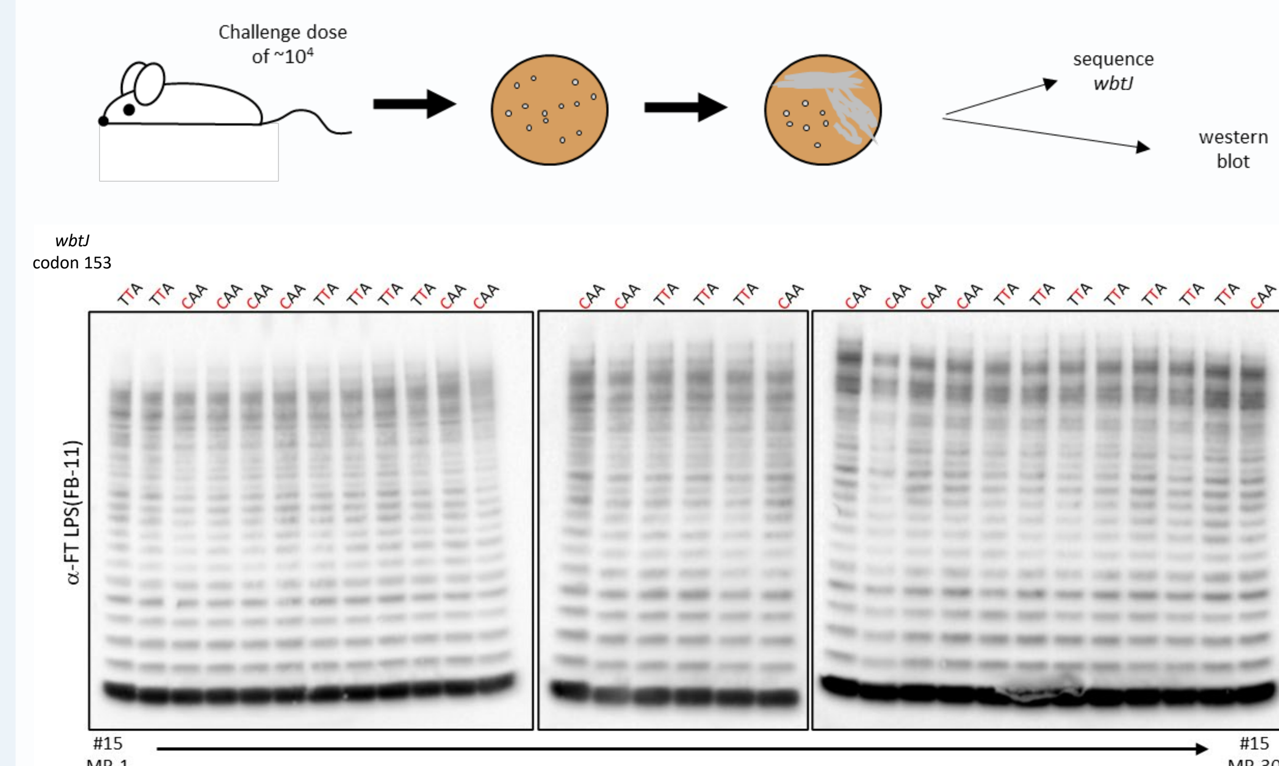


Figure 5: Phenotypic assessment of colonies recovered from mouse spleen at 21 days post challenge for Isolate #15. Western analysis with α-LPS on mouse passaged samples from isolate #15. The sequence at the codon mutated in *wbtJ* is indicated above each lane.

Discussion

We found that a strong selective pressure exists to select for reversion events when multiple colony morphologies were identified in macrophage lysates and, more importantly, *in vivo* when determining the bacterial burden of spleen homogenates. While the complete genome of the reverted isolates was not sequenced, our data are the first to confirm that phase variation occurs due to spontaneous mutations and reversion as a result of a secondary mutation masking the previous phenotype. A caveat to this study is that we relied upon isolate #15, which harbors a SNP, to monitor reversion. In this study, we consistently identified 3 forms of the repaired *wbtJ* allele (encoding a ser, leu or gln) for isolate #15 and wondered if saturation was reached for the repaired alleles. However, other potential mutations that could arise at this codon would result in tyrosine (aromatic), lysine or glutamine (charged), or another stop codon. With this in mind, we suspect that for isolate #15 the reverted isolates we identified are the most likely alleles that would repair WbtJ enzymatic function, especially considering codon 153 is near the region that interacts with substrate. It is likely that more deleterious mutations, such as deletion of multiple nucleotides, may be more difficult to repair and likely explain why some variants appear more stable than others (4). Construction of a locked variant is crucial to improving both the safety and efficacy of live vaccine strains of *F. tularensis.* Ideal candidates are thought to be variants locked in blue form (5, 6). However, it is possible that a particular gray form may be able retain the immunogenic properties necessary for vaccination and display high levels of attenuation as a systematic study of all the mutants responsible for phase variation has yet to be completed.

Summary

Using colony morphology as an indicator of variant state, gray to blue state reversion events were captured. Analysis of reverted isolates suggests that a secondary mutation near the original lesion can functionally repair the gene resulting in an otherwise wild-type (blue) state. While the exact mechanism of phase variation remains elusive, this study is the first to provide a genetic insight into the molecular mechanisms of blue/gray phase variation in *F. tularensis*.

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Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011 and The United States Army Medical Research Institute of Infectious Diseases Institutional Animal Care and Use Committee. The protocol was approved by the United States Army of Medical Research Institute of Infectious Diseases under an Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

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