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Original article

The contribution of the glycine cleavage system to the pathogenesis of *Francisella tularensis*

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Abstract

Biosynthesis and acquisition of nutrients during infection are integral to pathogenesis. Members of a metabolic pathway, the glycine cleavage system, have been identified in virulence screens of the intracellular bacterium *Francisella tularensis* but their role in pathogenesis remains unknown. This system generates 5,10-methylenetetrahydrofolate, a precursor of amino acid and DNA synthesis, from glycine degradation. To characterize this pathway, deletion of the *gcvT* homolog, an essential member of this system, was performed in attenuated and virulent *F. tularensis* strains. Deletion mutants were auxotrophic for serine but behaved similar to wild-type strains with respect to host cell invasion, intracellular replication, and stimulation of TNF-α. Unexpectedly, the glycine cleavage system was required for the pathogenesis of virulent *F. tularensis* in a murine model. Deletion of the *gcvT* homolog delayed mortality and lowered bacterial burden, particularly in the liver and bloodstream. To reconcile differences between the cell culture model and animal model, minimal tissue culture media was employed to mimic the nutritionally limiting environment of the host. This reevaluation demonstrated that the glycine cleavage system contributes to the intracellular replication of virulent *F. tularensis* in serine limiting environments. Thus, the glycine cleavage system is the serine biosynthetic pathway of *F. tularensis* and contributes to pathogenesis in vivo.

Keywords: *Francisella tularensis*; Glycine cleavage system; Host–pathogen interactions; Metabolism; Pathogenicity; Bacterial infections

1. Introduction

Francisella tularensis is an intracellular bacterium and a formidable pathogen. It is highly infectious, requiring inhalation of only 10–50 bacteria to cause a febrile illness known as tularemia [1]. The pulmonary manifestation of the disease is fatal in up to 60% of cases without medical intervention [2]. Due to these properties, there is a significant concern for intentional aerosolized release and misuse of this agent in the form of bioterrorism [3]. As such, *F. tularensis* is categorized by the Center for Disease Control and Prevention as a tier one select agent [4].

Often referred to as a "stealth pathogen", *F. tularensis* is capable of both suppressing and avoiding the host immune response [5]. Infection with *Francisella* evokes little to no proinflammatory response in vitro and a delayed proinflammatory response in vivo [6]. While eluding detection, this bacterium has a complex intracellular life cycle involving invasion, phagosomal escape, cytosolic replication, and egress [7]. Significant questions remain regarding the host pathogen interaction throughout its life cycle, but it is clear that *Francisella* is well suited for its intracellular niche. In support of this, *F. tularensis* is capable of successful infection and replication in an extensive repertoire of host cells. This repertoire ranges from immune cells such as dendritic cells, neutrophils, and macrophages to non-immune cells such as

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hepatocytes and type II pneumocytes [8,9]. Thus, *Francisella* is capable of circumventing host defense systems and gaining access to the cytosolic environment.

Organisms must acquire or synthesize various metabolites in order to survive and replicate. For pathogens, metabolites and metabolic precursors must be derived from the host. *Francisella* infects a wide range of host sites including the lung, liver, spleen, and blood [10]. The bacterium must therefore be metabolically competent for these nutritionally diverse environments. In support of this, tryptophan biosynthesis in *F. tularensis* has been found to be essential in counteracting lung specific inducible tryptophan starvation involving host production of indoleamine 2,3-dioxygenase [11]. Furthermore, the extracellular phase of this bacterium relies on a potassium uptake protein known as TrkH to grow in the potassium-limiting environment of the host's blood [12]. Cell type specific nutritional requirements have also been discovered as pyrimidine biosynthesis is required for replication in macrophages but not in epithelial cells [13]. In contrast, purine biosynthesis is important to *Francisella* intracellular replication across cell types and loss of this pathway results in a dramatic attenuation in vivo [14]. Thus, investigation into pathogen metabolism during infection has revealed important pathways contributing to *F. tularensis* pathogenesis. Broadly, these results have also added to a growing understanding of the microenvironments in host tissues and the biosynthetic and nutrient acquisition pathways that are critical for pathogens to colonize these niches.

Despite recent advances, a significant number of metabolic pathways remain uncharacterized in *F. tularensis* and their contribution to pathogenesis is unknown [15]. One particular unstudied pathway, the glycine cleavage system (GCS), has a variety of noteworthy properties. This system facilitates the degradation of glycine to acquire 5,10-methylene-tetrahydrofolate, a one carbon donor utilized in the production of serine, thymidine, and purines [16]. Therefore, this pathway is expected to contribute to pathogen fitness in host compartments where these metabolites, such as serine, are limiting. The homologs of the GCS are transcriptionally upregulated in *F. tularensis* during infection of macrophages [17]. A member of this system, the homolog of *gcvH*, was found to be strongly induced at the protein level in *Francisella* isolated from mouse spleens [18]. Furthermore, this system was identified in an in vivo negative selection screen in the related bacterium, *Francisella novicida* [19]. These data suggest that the glycine cleavage system may play an important role in the metabolic fitness of *F. tularensis*.

In this report, we evaluated the contribution of the glycine cleavage system to the pathogenesis of *F. tularensis.* To investigate this pathway, we deleted the *F. tularensis* homolog of a required member of this system, the glycine cleavage protein T (*gcvT*). Following mutagenesis, strains were assayed for metabolic defects, in vitro virulence phenotypes, and in vivo pathogenesis. Our results demonstrate that the homolog of the glycine cleavage system is functional, essential when serine is limited, and ultimately required for the full in vivo pathogenesis of virulent *F. tularensis.*

2. Materials and methods

2.1. Bacterial strains

The following reagent was obtained through BEI Resources, NIAID, NIH: *F. tularensis* subsp. *tularensis,* Strain SCHU S4 (FSC237), NR-643. *F. tularensis* subsp. *holarctica* Live Vaccine Strain (LVS) was provided as a gift from Dr. Karen Elkins (U.S. Food and Drug Administration). Routine culture of *Francisella* was performed by streaking frozen bacterial stocks onto chocolate agar (GC medium base, hemoglobin, and isovitaleX). Bacteria were grown on plates for three days at 37 °C with 5% CO_2 and subsequently used to inoculate overnight cultures. Unless otherwise specified, overnight cultures were performed in trypticase soy broth supplemented with cysteine (TSB-C) and were shaken at 250 rpm at 37 °C. All work with Schu S4 strains was performed in BSL3 containment with approval from the Centers for Disease Control and Prevention Select Agent Program. Cloning was performed using the *Escherichia coli* EC100D strain.

2.2. Generation of deletion mutants, complements, and vector controls

Deletion of the *gcvT* homolog in LVS (FTL_0477) and Schu S4 (FTT_0407) was performed by allelic replacement as described previously [20]. The flanks of *gcvT* share 99% identity between LVS and Schu S4, therefore we utilized a suicide vector (pJH1) containing the regions 1000 bp upstream and downstream of *gcvT* in LVS for deletions in both strains. The upstream flank was generated using CATGGGATCCCCGATAGTTGCTAGCGTGG as a forward primer and GATCGGTACCTGCTATATGTGATTCATAAAGAGG as a reverse primer. The downstream flank was generated using CTAGGGTACCGTCGAGCTAGTTAAACCTAAG as a forward primer and CTAGGCATGCCTCTCAAATAAGTTGGGTGTAAAGC as a reverse primer. Loss of *gcvT* was confirmed by genomic PCR using CTAGGGATCCGCTACCAACTTTATATGCGGAAGATCC as a forward primer and CTAGGGTACCTGACCCACTCATGCGACTTTGTATAC as a reverse primer. Strains with deletions of the *gcvT* homolog are annotated as LVS Δ*gcvT* and Schu S4 Δ*gcvT.*

Complementation of *gcvT* was performed in cis using a modified pJH1 suicide plasmid (pMB1). pMB1 was generated by cutting pJH1 with XhoI to remove extraneous yeast genes and subsequent religation. The LVS *gcvT* (FTL_0477) was amplified by PCR with a ~250 bp of upstream region and ligated into pGEM-T, generating pGEM*gcvT.* This PCR product was generated using GATCGGATCCCCTGGAGAGAAGATAACCGAAGAATC as a forward primer and CTAGGCATGCTGACCCACTCATGCGACTTTGTATAC as a reverse primer. Digestion of pGEM*gcvT* with BamHI and SphI (restrictions sites present in the forward and reverse primers) allowed for subcloning of this sequence into pMB1 to create pMB1*gcvT.* This procedure was repeated with a PCR amplicon containing only the 250 bp upstream region to serve as a pMB1 vector control. This PCR product was generated