The Role of Rv2623 in Deciphering the Mechanisms of Tuberculosis Latency

By: Amulya Marellapudi

Nyack High School

Abstract:

Mycobacterium tuberculosis (Mtb), the bacterium that causes tuberculosis (TB), can hide in an infected host in a latent form without causing symptoms. Latent Mtb can subsequently reactivate to manifest the disease and thus transmission of the bacillus. Since it has been estimated that one-third of the world's population harbors latent Mtb, understanding how this bacterium becomes latent and subsequently reactivates can lead to better TB control.

M. tuberculosis universal stress protein (USP) Rv2623 was chosen for this study because it is one of the most highly induced genes in the dormancy regulon when bacilli are subjected to hypoxia and nitrosative stress. More importantly, Rv2623 was also shown to be up-regulated when the tubercle bacillus is internalized by human and mouse macrophages as well as in the lungs of mice with persistent *M. tuberculosis* infection. These observations suggest that the induction of Rv2623 may have biological relevance. Taking a multidisciplinary approach involving genetics, biochemistry, and immunology, results have been provided suggesting that Rv2623 modulates bacterial growth by regulating the function of Rv1747, a putative exporter of a family of mycobacterial Mtb glycolipids that can modulate the immune response.

Understanding the Rv2623-Rv1747 interaction should illuminate how Mtb enters latency. A better understanding of the mechanisms of tuberculosis latency is integral for the development of anti-TB vaccines.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), remains an enormous public health burden (WHO, 2015). It has been estimated that in 2014, 1.5 million individuals were killed by the tubercle bacillus, and there were 9.6 million new cases worldwide (WHO, 2015). A unique feature of *M. tuberculosis* is its ability to establish a quiescent, clinically silent, latent infection in the host, which can subsequently reactivate to cause the disease to become active (Chan, 2004; Gomez, 2004). In immunocompetent individuals who harbor a latent infection, the lifetime risk for developing reactivation of TB is 10% (Figure 1). This risk increases to 10% per year in the setting of co-infection with the human immunodeficiency virus (HIV). Since one-third of the world's population have been infected with *M. tuberculosis* (WHO, 2015), and the majority of these individuals harbor latent bacilli that can recrudesce, the latently infected constitute a major reservoir for the perpetuation and transmission of TB (Chan, 2004; Gomez, 2004). Understanding the mechanisms of tuberculosis latency and reactivation is therefore of paramount importance for the development of effective strategies for the control of TB. Despite the significance of tuberculosis latency in TB pathogenesis, the mechanisms underlying this process remain incompletely defined (Chan, 2004; Gomez, 2004).

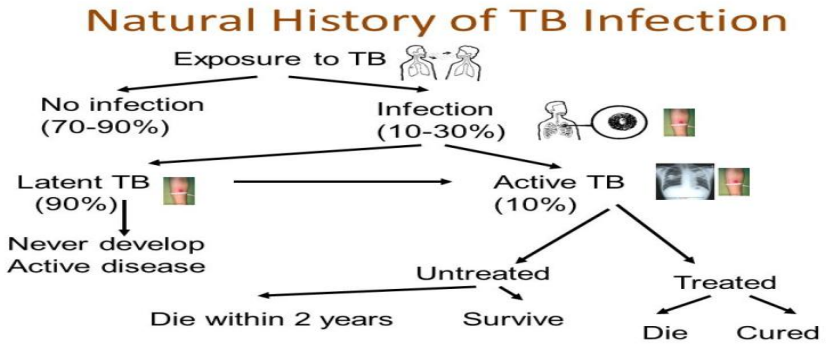


Figure 1: Progression of Tuberculosis in immunocompetent individuals
("Tuberculosis An Old Disease – New Twists A Continuing Public Health Challenge Jane Moore, RN, MHSA Director, TB Control & Prevention Program 2012 EPID.")

It has been previously observed that Rv2623, an *M. tuberculosis* universal stress protein (USP), regulates bacterial growth both *in vitro* and *in vivo* (Drumm, 2009). *M. tuberculosis* USP Rv2623 was studied because it is one of the most highly induced genes in the dormancy regulon when bacilli are subjected to hypoxia and nitrosative stress. More important, Rv2623 was also shown to be up-regulated when the tubercle bacillus is internalized by human and mouse macrophages. In *in vitro* systems, over expression of Rv2623 in *M. tuberculosis* and in the relatively avirulent *M. smegmatis* (strain mc^2155) results in growth retardation in recipient bacteria (Drumm, 2009). In animal models, deletion of Rv2623 by specialized transduction results in a mutant, ΔRv2623, that is hypervirulent and unable to establish a chronic persistent infection (Drumm, 2009). Biochemical and structural studies have revealed that Rv2623 is an ATP-binding protein (Drumm, 2009). Based on a 2.9-Å-resolution crystal structure, mutants with reduced capacity to bind ATP were obtained (Drumm, 2009). Analyses of these mutants have revealed that the ability of Rv2623 to regulate growth of *M. tuberculosis* is dependent on the capacity of the USP to bind ATP (Drumm, 2009). It was previously reported by Drumm in 2009 that Rv2623, which encodes a USP and is highly induced by hypoxia and nitrosatrive stress, can regulate bacillary growth *in vitro* and *in vivo*. Based on these observations, it is postulated that Rv2623 contributes to the establishment of persistent latent infection in *M. tuberculosis*-infected individuals (Drumm, 2009). Thus, elucidation of the how Rv2623 regulates growth may illuminate mechanisms underlying tuberculosis latency and reactivation.

Current studies seek to characterize the mechanisms by which Rv2623 regulates the growth of *M. tuberculosis*. A multi-disciplinary approach, which involves methods used in genetics, biochemistry, bioinformatics, and microbiology, has been taken to address this issue. The results of these studies have provided evidence suggesting that: (i) Rv2623 interacts with Rv1747, a mycobacterial protein that has been annotated as a putative ABC transporter that exports lipooligosaccharides (Braibant, 2000), to negatively regulate *M. tuberculosis* growth; (ii) this interaction is enabled by the recognition of a conserved phosphothreonine-containing oligopeptide motif of Rv2623 by the FHAI domain of Rv1747 (Durocher, 2002; Pallen, 2002); and (iii) ΔRv2623 displays a morphological phenotype that is distinct from that of wild-type (WT) *M. tuberculosis*, and is associated with increased levels of phosphatidyl-*myo*-

inositol mannosides (PIMs), immunologically active molecules that can modulate the host immune response to the tubercle bacillus (Guerin, 2010; Torrelles, 2010). By contrast, an Rv1747-deletion mutant, compared to WT bacilli, produces less PIMs. Of note, while ΔRv2623 has been reported to be hypervirulent in mice and guinea pigs (Drumm, 2009), an Rv1747 deletion *M. tuberculosis* mutant is hypovirulent in mice (Curry, 2005; Spivey, 2011). The results to be determined by the present study should provide a useful platform that enables further characterization of the mechanisms that regulate the growth of *M. tuberculosis*, particularly in the context of establishing tuberculous latency.

Methodology:

Bacterial strains, culture conditions, and plasmids

M. tuberculosis strain Erdman and H37Rv and *M. smegmatis* mc^2155 were cultured in supplemented Middlebrook 7H9 medium (Becton Dickinson, Sparks, MD). Mycobacterial strains overexpressing WT Rv2623 and various mutants of the USP were maintained in supplemented 7H9 media containing 40 μg/ml of kanamycin. Mycobacterial growth was monitored by measuring absorbance at 600 nm or by the BACTEC 9000 system. The latter approach involved inoculating stationary phase *M. tuberculosis* or *M. smegmatis* in triplicates into BD Myco/F lytic vials (final bacterial suspension of 10^4 colony forming units (CFU) per ml), whose liquid medium is supplemented with a compound that fluoresces as a result of oxygen depletion due to bacterial growth. The time to detection of fluorescence signals thus reflected the rate of bacterial growth.

In vitro phosphorylation of Rv2623

Equimolar concentrations of recombinant Rv2623 (expressed in and purified from either *E.coli* or *M. smegmatis* as described above) were incubated in the presence of 100 μM ATP (Sigma Aldrich) in kinase reaction buffer (25 mM HEPES pH 7.4, 20 mM $MgCl_2$, 20 μM $MnCl_2$, 1 mM DTT) at room temperature for different time points. The reaction was stopped by addition of 6X SDS dye and heat inactivated at 65°C for 10 minutes. Samples

were electrophoretically resolved in a 10% SDS PAGE, transferred onto a PVDF membrane, and probed with an mAb against phosphothreonine and then allowed to react with an appropriate HRP-conjugated secondary antibody. Signals were detected by the Amersham ECL Plus Chemiluminescence kit. Rv2623 protein was identified by molecular mass.

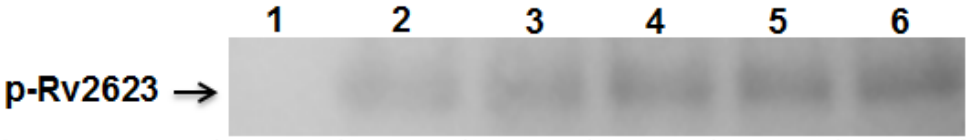
Results:

M. tuberculosis proteins were cloned and expressed. In total, four constructs were generated to express the recombinant *M. tuberculosis* Rv1747 FHAI domain, a protein signaling module that mediates protein-protein interaction through binding to a conserved oligopeptide motif containing a phosphothreonine of its interactor .

Figure 2: *M. tuberculosis* Rv1747.

The primary structure of Rv1747 (it has been annotated to be an ABC transporter that export lipooligosaccharides with 2 FHA domains (FHA I & FHA II); elements typical of ABC transporters: NBD (*n*ucleoside-*b*inding *d*omain; 1-559 amino acids (a.a.); Walker A&B (the ATP-binding domain), and transmembrane domain. The FHAI domain spans amino acid 29-92. *M. tuberculosis* Rv1747 contains all domains typical of an ABC transporter.

Next, Rv2623 was post-translationally modified and harbored phosphorylated threonines that interacted with Rv1747. To further evaluate the phosphorylation status of Rv2623, the USP, purified from *M. tuberculosis* and BCG lysates using the Rv2623-specific monoclonal antibody, were subjected to Western blot analysis.



Reaction Time (min)	60	5	15	30	45	60
Rv2623 (*E. coli*)	+	+	+	+	+	+
PknG	-	+	+	+	+	+

Reaction Time (min)	60	5	15	30	45	60
Rv2623 (Msmeg)	+	+	+	+	+	+
PknG	-	+	+	+	+	+

Figure 3: Results of 2-D gel electrophoretic analyses of Rv2623 and its reactivity with anti-phosphothreonine antibodies

Finally, potentially phosphorylated threonine residues of Rv2623 were identified**.** To obtain adequate amounts of phosphorylated Rv2623 for mass spectrometric analysis, the *M. tuberculosis* serine threonine protein PknG was used to phosphorylate purified recombinant Rv2623 in vitro. The phosphorylated samples were electrophoretically resolved by SDS-PAGE. To evaluate if PknG was able to phosphorylate Rv2623, the resolved proteins were transferred onto nitrocellulose membrane. Western Blot analysis of the membrane using an anti-phosphothreonine monoclonal antibody and an anti-Rv2623 monoclonal antibody revealed that the USP was successfully phosphorylated (Figure 3). The results showed that Rv2623 reactivity to the anti-phosphothreonine monoclonal antibody increased with time as the kinase reaction progressed (Figure 3).

Together, these results have provided compelling evidence that specific elements of Rv2623 and the FHAI domain of Rv1747 mediate the interaction between these two mycobacterial components to negatively regulate *M. tuberculosis* growth in a phosphorylation-dependent manner.

Conclusion:

The present study sought to understand the mechanisms by which Rv2623 regulates *M. tuberculosis* growth. The results have provided evidence that this USP interacts with Rv1747, a putative ABC lipooligosaccharide exporter, to regulate *M. tuberculosis* growth in concert. This interaction is mediated via the recognition by the Rv1747 FHAI and the Rv2623 conserved oligopeptide motif harboring a phosphorylated threonine at position 237.

Taken together, the interaction of Rv2623 with Rv1747 negatively modulates the growth of virulent *M. tuberculosis.* The discrepant *in vivo* virulence and PIM expression phenotypes of ΔRv2623 and Δ*Rv1747* suggest that the USP may regulate mycobacterial growth by moderating the functional activity of Rv1747, implying that the ABC transporter could function to export PIM(s).

In summary, the present study has provided evidence that *M. tuberculosis* Rv2623 negatively modulates the function of Rv1747 to regulate mycobacterial growth through phosphorylation-dependent mechanisms. Further, the opposing PIM expression phenotype of these two mycobacterial components suggests that Rv1747 is a transporter for PIMs, immunologically active molecules that have been shown *in vitro* to impact *M. tuberculosis*-host interaction to influence the immune response. The immune-regulatory properties contribute, at least in part, to the ability of the Rv2623-Rv1747 interaction to regulate *M. tuberculosis* growth in the host. PIMs are down regulated in their stationary phase, and it has recently been observed that *M. tuberculosis* in primary human macrophages enhances PIM expression. Much remains to be learned with respect to the precise mechanisms by which Rv2623 regulates mycobacterial growth.

Finally, the apparent function of Rv1747 as a PIM transporter and the mechanisms underlying its regulation remain to be characterized. This will likely not be a straightforward endeavor given the transmembrane nature of Rv1747. Nevertheless, the collective results of the present study have provided a framework for understanding the mechanisms by which Rv2623

interacts with Rv1747 to regulate *M. tuberculosis* growth, particularly in the context of tuberculosis persistence, and for advancing the knowledge of the biosynthetic pathways of mycobacterial glycolipids and lipoglycans. The data strongly suggest Rv2623 is a critical component that regulates the entry of *M. tuberculosis* into a chronic persistent growth phase, and therefore provides valuable insight into tuberculosis dormancy and uncovers new opportunities for the development of novel anti tuberculosis therapies.

Bibliography:

Braibant, M. "The ATP Binding Cassette (ABC) Transport Systems of Mycobacterium Tuberculosis." *FEMS Microbiology Reviews* 24.4 (2000): 449-67. Web.

Chan, John, and Joanne Flynn. "The Immunological Aspects of Latency in Tuberculosis." *Clinical Immunology* 110.1 (2004): 2-12. Web.

Curry, J. M., R. Whalan, D. Hunt M., K. Gohil, M. Strom, L. Rickman, M. Colston J., S. Smerdon J., and R. Buxton S. "An ABC Transporter Containing a Forkhead-Associated Domain Interacts with a Serine-Threonine Protein Kinase and Is Required for Growth of Mycobacterium Tuberculosis in Mice." *Infection and Immunity* 73.8 (2005): 4471-477. Web.

Drumm, Joshua E., Kaixia Mi, Patrick Bilder, Meihao Sun, Jihyeon Lim, Helle Bielefeldt-Ohmann, Randall Basaraba, Melvin So, Guofeng Zhu, Joann Tufariello M., Angelo Izzo A., Ian Orme M., Steve Almo C., Thomas Leyh S., and John Chan. "Correction: Mycobacterium Tuberculosis Universal Stress Protein Rv2623 Regulates Bacillary Growth by ATP-Binding: Requirement for Establishing Chronic Persistent Infection." *PLoS Pathog PLoS Pathogens* 5.9 (2009): n. pag. Web.

Durocher, Daniel. "The FHA Domain." *CESARENI:PROTEIN DOMAINS O-BK Modular Protein Domains* (2005): 143-62. Web.

Gomez, James E., and John McKinney D. "M. Tuberculosis Persistence, Latency, and Drug Tolerance." 29-44 84.1-2 (2004): n. pag. Web.

Guerin, M. E., J. Kordulakova, P. Alzari M., P. Brennan J., and M. Jackson. "Molecular Basis of Phosphatidyl-myo-inositol Mannoside Biosynthesis and Regulation in Mycobacteria." *Journal of Biological Chemistry* 285.44 (2010): 33577-3583. Web.

Pallen, Mark, Roy Chaudhuri, and Arshad Khan. "Bacterial FHA Domains: Neglected Players in the Phospho-threonine Signalling Game?" *Trends in Microbiology* 10.12 (2002): 556-63. Web.

Spivey, V. L., V. Molle, R. Whalan H., A. Rodgers, J. Leiba, L. Stach, K. Walker B., S. Smerdon J., and R. Buxton S. "Forkhead-associated (FHA) Domain Containing ABC Transporter Rv1747 Is Positively Regulated by Ser/Thr Phosphorylation in Mycobacterium Tuberculosis." *Journal of Biological Chemistry* 286.29 (2011): 26198-6209. Web

Torrelles, Jordi B., and Larry Schlesinger S. "Diversity in Mycobacterium Tuberculosis Mannosylated Cell Wall Determinants Impacts Adaptation to the Host." *Tuberculosis* 90.2 (2010): 84-93. Web.

WHO. WHO Report 2015: Global Tuberculosis Control. (2015). Web.