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ICAT-based comparative proteomic analysis of non-replicating persistent *Mycobacterium tuberculosis*

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Summary The non-replicating persistence (NRP) phenotype of *Mycobacterium tuberculosis* (NRP-TB) is assumed to be responsible for the maintenance of latent infection and the requirement of a long treatment duration for active tuberculosis. Isotope coded affinity tag-based proteomic analysis was used for the determination of the relative expression of large numbers of *M. tuberculosis* proteins during oxygen self-depletion under controlled conditions in a multi-chambered fermentor. Expression of the α-crystallin homolog protein, acr, was monitored and quantified to confirm entry into NRP. Relative expression of 586 and 628 proteins was determined in log phase vs. early stage NRP (NRP-1) and log phase vs. later stage NRP (NRP-2), respectively. Relative to expression in log phase and using an abundance ratio of ± 2.0 as a cutoff, 6.5% and 20.4% of proteins were found to be upregulated in NRP-1 and NRP-2, respectively while 20.3% and 13.4% were downregulated, respectively. Functional profiling revealed that 42.1%/39.8% of upregulated proteins and 41.2%/45.2% of downregulated proteins in NRP-1/NRP-2, respectively, were involved in small molecule metabolism. Among those proteins the highest proportions of 37.5% in NRP-1 were involved with degradation and of 45.1% in NRP-2 with energy metabolism. These results suggest distinct protein expression profiles in NRP-1 and NRP-2.

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Introduction

There is a general consensus [1] that the ideal new drug for tuberculosis would (a) reduce the duration of treatment from the current minimum of 6 months to 2 or 3 months (which in turn would

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reduce the development of acquired drug resistance since most patients who fail to complete therapy default after this time), (b) be active against drug-resistant strains and (c) be capable of eradicating latent TB infection. It is generally accepted that a physiological state characterized by maintained viability but no growth (non-replicating persistence; NRP) and resulting in reduced drug susceptibility is responsible for the requirement for long treatment duration in TB.[2,3] Therefore a comprehensive understanding of the cellular processes occurring during NRP should facilitate attempts to interfere with this process in a rational manner.

In vitro conditions that induce the NRP stage in *Mycobacterium tuberculosis* include late stage stationary growth and/or hypoxic conditions. It was recognized early by Wayne and colleagues that these conditions induce a shift to the glyoxylate pathway for energy generation.[4] The Wayne model, in which oxygen limitation is the growth-limiting factor, has become the standard model for NRP *M. tuberculosis*.[5] Further studies defined two stages of metabolic adaptation, the first to microaerophilic conditions (NRP stage 1, NRP-1), and the second to essentially anaerobic conditions (NRP stage 2, NRP-2).[6] A subsequent study confirmed the importance of the glyoxylate pathway for *M. tuberculosis* persistence in macrophages and in mice by knocking out the branch enzyme isocitrate lyase.[7] Others have used the Wayne model to identify genes and proteins induced in *M. tuberculosis* and *Mycobacterium bovis* BCG and *M. smegmatis* during oxygen limitation.[8–13] Among several proteins identified as being highly expressed in *M. tuberculosis* under low-oxygen conditions, induction of an α-crystallin protein homolog (Acr, HspX, Rv2031c) has been the most intensively investigated.[4,8,13–20]

In the Wayne model, *M. tuberculosis* cells are subjected to self-depletion of oxygen by cultivation in sealed batch cultures. However, poorly defined batch cultures cannot be investigated for functional proteomics studies. These cultures are comprised of physiologically heterogeneous subpopulations and bear little resemblance to the state of bacteria in the dynamic host environment.[21] Some parameters such as oxygen tension and restricted iron availability play an important role in microbial virulence gene expression and are likely to play a key role in regulating the pathogenicity of *M. tuberculosis*.[22] Several in vitro model systems have been developed to examine the protein level response of *M. tuberculosis* to environmental stress and intracellular residence within macrophages.[23–26] Additional studies have used stationary culture,[9,18,27] standing and submerged culture,[3,8,28] and shallow standing culture system to mimic oxygen limitation environment.[14,17] By contrast, chemostat culture allows bacteria to be grown in controlled and defined environment at a constant rate. Using this technique, growth parameters can be monitored and manipulated independently. *M. tuberculosis* has been successfully grown in a chemostat culture system.[29]

The conventional quantitative method of measuring changes of protein expression level adapts two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)[30] and generally utilizes a comparison of protein spot intensities.[31–33] This strategy suffers from limitations in protein coverage, sensitivity, dynamic range, and precision of measurements.[34,35] It is also difficult to compare 2-D PAGE results from different laboratories despite increased reproducibility through standardization and automation.[36,37] An alternate approach circumvents gel electrophoretic protein separation in favor of direct MS analysis via so-called shotgun proteomic methods such as ICAT[38] and MUDPIT.[39] The ICAT reagent strategy is predicated upon distinguishing between two protein populations labeled with two different stable isotope tags (d0: light chain, d8: heavy chain).[38] The protein mixtures are combined, then digested with the enzyme trypsin, and the ICAT reagent labeled peptides are isolated by avidin-affinity chromatography using the biotin group on the reagent as an affinity tag. The amino acid sequence of a peptide is determined from tandem mass spectra of individual ICAT-labeled peptides correlated with amino acid sequences in a database. The relative abundance of a protein from which the peptide originated is compared by the ratio of signal intensities of isotopically normal and heavy chain labeled peptide pairs.[27,40–43] One significant obstacle to the routine implementation of ICAT method is that proteolytic digests of cells are exceedingly complex, with literally hundreds of thousands of peptides generated in a sample.[43] To help alleviate this problem current ICAT strategy separates ICAT-labeled peptides on a strong-cation exchange (SCX) column prior to avidin column isolation of ICAT-labeled peptides. This ICAT approach has been successfully applied to many different cell types and revealed changes in protein expression as a result of various perturbations.[42–46] Recently, comparative 2-D PAGE analysis of *M. tuberculosis* during aerobic and anaerobic culture[13] and ICAT-2D PAGE analysis for *M. tuberculosis* vs. *M. bovis* BCG proteomes showed differentially expressed proteins.[47]