A Chemical Proteomics Approach to Profiling the ATP-binding Proteome of Mycobacterium tuberculosis*

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Tuberculosis, caused by Mycobacterium tuberculosis, remains one of the leading causes of death worldwide despite extensive research, directly observed therapy using multidrug regimens, and the widespread use of a vaccine. The majority of patients harbor the bacterium in a state of metabolic dormancy. New drugs with novel modes of action are needed to target essential metabolic pathways in M. tuberculosis; ATP-competitive enzyme inhibitors are one such class. Previous screening efforts for ATP-competitive enzyme inhibitors identified several classes of lead compounds that demonstrated potent anti-mycobacterial efficacy as well as tolerable levels of toxicity in cell culture. In this report, a probe-based chemoproteomic approach was used to selectively profile the M. tuberculosis ATP-binding proteome in normally growing and hypoxic M. tuberculosis. From these studies, 122 ATP-binding proteins were identified in either metabolic state, and roughly 60% of these are reported to be essential for survival in vitro. These data are available through ProteomeXchange with identifier PXD000141. Protein families vital to the survival of the tubercle bacillus during hypoxia emerged from our studies. Specifically, along with members of the DosR regulon, several proteins involved in energy metabolism (Icl/Rv0468 and Mdh/Rv1240) and lipid biosynthesis (UmaA/Rv0469, DesA1/Rv0824c, and DesA2/Rv1094) were found to be differentially abundant in hypoxic versus normal growing cultures. These pathways represent a subset of proteins that may be relevant therapeutic targets for development of novel ATP-competitive antibiotics.

Tuberculosis remains a significant global health burden, and the emergence of multidrug-resistant and extensively drug-resistant cases continue to increase (1). Thus novel chemotherapy for the treatment of drug-resistant disease are needed. In addition, antibiotics that reduce the effective time (>6 months) and complexity of antibiotic regimens used (three to four drugs in tandem) are needed for more effective treatment of Mycobacterium tuberculosis. The recent description of ATP-competitive enzyme inhibitors as a novel class of antitubercular drugs (2–5) has bolstered interest in the identification of bacterial enzymes that utilize ATP as these enzymes may be essential and druggable targets for the discovery and design of such small molecule inhibitors. Furthermore, elucidating ATP-dependent catalytic pathways present in differing metabolic disease states is critical for understanding mechanisms of latency, virulence, and pathogenesis. This study and others (6) lay the groundwork for profiling of the ATPome across diverse infectious diseases under different metabolic states that may be relevant within the host milieu, with the goal of identifying critical and potentially druggable ATP-dependent pathways. For noninfectious diseases, a recent study utilized activity-based chemoproteomic profiling in murine models of induced obesity to study metabolic changes associated with mitochondrial dysfunction (7). For M. tuberculosis, manipulation of these critical signaling pathways via novel chemotherapeutic strategies could not only increase the effectiveness of drug treatment in multidrug-resistant/extensively drug-resistant cases but may also enhance efficacy in vivo against bacilli exhibiting multiple and often resistant phenotypes within the host (8). The study of kinases and other ATP-binding proteins (chaperones, ATPases, synthases, and other metabolic enzymes) has become important in elucidating the roles of ATP-dependent pathways in the pathogenesis of cancer and other mechanisms of dysregulated growth. The large scale profiling
of such networks is facilitated with the use of active-site nucleotide capture probes (9, 10). Traditionally, studies have utilized this chemical proteomics approach to map cellular interaction networks of protein kinase inhibitors as well as to elucidate global protein kinase profiles of cell lines (10–12). Here, we describe a chemical proteomics method that is designed to capture the full array of adenosine nucleotide-binding proteins, or the ATPome, of *M. tuberculosis* H37Rv. This method utilizes a desthiobiotin-conjugated ATP as a molecular probe in which target enzymes are covalently modified with biotin within characteristic active sites, in this case the nucleotide binding domains of kinases and other ATP-binding proteins. Once labeled, ATP-binding proteins are subsequently digested with trypsin and labeled peptides enriched via streptavidin affinity capture beads and subjected to LC-MS/MS for the identification of ATP-labeled proteins. The utility of this approach is multifaceted; the profiling of inhibitor selectivity in native proteomes can be achieved quickly and without the need for radiolabeling, recombinant enzymes, and functional assays. Additionally, the differential abundances of ATP-binding proteins during different growth states and conditions can be selectively monitored and quantified. Thus, this technology can be broadly applied to emerging infectious diseases and/or select agents where few other tools are readily available for drug discovery. Here, we identified essential gene products critical to survival, adaptation, and the development of drug resistance in *M. tuberculosis*. These results may lead to the identification and facile monitoring of novel therapeutic targets and their interactions within pathogen-specific pathways.

**EXPERIMENTAL PROCEDURES**

**Bacterial Growth—** *M. tuberculosis, H₃₇Rv* seed culture was grown to log phase (**Aₖ₇₀ 1.2**) in Middlebrook 7H9, ADC. Normally grown cultures were as follows. Three cultures (195 ml in 500-ml vented cap flasks) were inoculated with 2.5 ml of seed culture. A magnetic stir bar was added, and cultures were incubated at 37°C with stirring (200 rpm) to a final **Aₖ₇₀ 1.2–1.6**. For hypoxic cultures, six cultures (paired replicates of 390 ml in 500-ml sealed cap flasks) were inoculated with 5.0 ml of seed culture. Cultures were incubated at 37°C with stirring (100 rpm) to a final **Aₖ₇₀ 0.65–0.70**. Cell pellets were harvested on days 7 (normal) and 14 (hypoxic).

**Sample Preparation—** Cell pellets were resuspended at a concentration of 0.5 g/ml in IP/Lysis Buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 5% glycerol) containing HALT™ protease/phosphatase inhibitor mixture (ThermoPierce). Resuspended cells were placed in Lysing Matrix B bead beater vials (pre-filled with 0.1 mm of silica; MP Biomedicals). Lysis of cells occurred over 12 bead-beat cycles (30 s lyse/45 s rest on ice). Cell lysates were cleared of silica and cellular debris via centrifugation at 4,000 × g for 10 min. Supernatant was transferred to a new microcentrifuge tube and centrifuged again for 10 min at 13,000 × g. Cleared lysate was then filtered through a 0.8/0.2-μm syringe filter to sterilize the lysate for working under BSL-2 conditions. The sterile lysate was desalted using 7K Thermo Scientific Zeba™ spin desalting columns, and the protein amount was quantified by BCA (ThermoPierce). 500-μg aliquots of whole cell lysate were labeled with 5 μM desthiobiotin-ATP for 10 min as per the manufacturer’s instructions (ThermoPierce).

**Active Site Peptide Capture—** Desthiobiotin-ATP-labeled proteins were reduced in 1 mM DTT and alkylated in 1 mM iodoacetamide before buffer exchange into digestion buffer (20 mM Tris, pH 8.0, 2 mM urea). Each sample was digested with trypsin (1 μg/μl) at an enzyme to substrate ratio of 1:50 for 2 h at 37°C. Peptide capture with streptavidin-agarose resin and elution using 50% acetonitrile, 0.1% TFA was followed as per the manufacturer’s instructions.

**LC-MS—** Peptides were separated on a nanospray column (Zorbax 300SB-C18, 3.5 μm, 75-μm inner diameter × 150-mm column (Agilent Technologies)). Samples were eluted into an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) using a gradient of 0–100% B (A = 3% ACN, 0.1% formic acid; B = 100% ACN, 0.1% formic acid) at a flow rate of 300 nl/min for 103 min. All samples were run in triplicate.

**Database Searching—** Tandem mass spectra were extracted, charge state deconvoluted, and deisotoped by Xcalibur version 2.2 SP1. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02) and SEQUEST (Thermo Fisher Scientific, San Jose, CA; version v.27, revision 11). Mascot and SEQUEST were set up to search the MtbRevers041712 database (7992 entries) assuming the digestion enzyme trypsin. Parameters for both search engines were set to a fragment ion mass tolerance of 1.0 Da and a parent ion tolerance of 2.5 Da. Oxidation of methionine, iodoacetamide derivative of cysteine, and the desthiobiotin modification of lysine were specified in Mascot and SEQUEST as variable modifications.

**Criteria for Protein Identification—** Scaffold (version Scaffold_3.6.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Mascot identifications required ion scores to be greater than the associated identity scores and 50, 65, 85, and 65 for singly, doubly, triply, and quadruply charged peptides. SEQUEST identifications required ΔCn scores of greater than 0.2 and XCorr scores of greater than 1.8, 2.0, 3.0, and 4.0 for singly, doubly, triply, and quadruply charged peptides. Protein identifications were accepted if they contained at least one identified peptide in at least two biological replicates. Peptide spectra meeting the minimum requirements were manually inspected for quality, using metrics described previously (13, 14). Quantification of proteins was performed on normalized spectral abundance factors for each protein (NSAF) (13–15). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. False discovery rates were calculated for each reported data set as follows: hypoxic versus normal (FDR = 13.1%), normal_ATP versus normal_ATP-S (FDR = 3.2%), hypoxic_ATP versus hypoxic_ATP-S (FDR = 11.2%), and the noncomparative ATPome (FDR = 6%). The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (16) with the dataset identifier PXD000141.

**Statistical Analysis—** The design for each experimental condition consisted of three biological replicates per sample group (normal, hypoxic, normal/hypoxic-STPK) and the noncomparative ATPome only. In the case of hypoxic cultures, six biological replicates were grown to set time points, and cell material was pooled into three-paired replicates and subsequently treated as triplicate replications.

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1 The abbreviations used are: NSAF, normalized spectral abundance factor; ATP₆S, adenosine 5′-[γ-thio]triphosphate tetralithium; BisTris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol; FC, fold change; USP, universal stress protein; FDR, false discovery rate; Icl, isocitrate lyase; STPK, serine/threonine kinase.
Each replicate was injected into the mass spectrometer three times for a total of nine injections per sample. Spectral count data, as visualized in Scaffold (Proteome software, version 3.6.1), were normalized to quantitative values using normalized spectral abundance factors, as described previously (13–15). Statistical analysis was performed using Fisher’s exact test in the comparison of two groups (i.e. normal-ATP versus hypoxic-ATP or normal-ATP versus normal-ATP). Fisher’s exact test is a valid method of identifying differences in protein abundance (i.e. spectral counts) in shotgun proteomics data sets using experimental designs of at least three biological replicates, and it performs with similar power to more complex generalized linear modeling strategies (17).

**Blast-based Sequence Description**—The most relevant description for each of the sequences was acquired based on the significant BLAST results. The homologs for the sequences were retrieved using the Blastp algorithm and the nonredundant database of NCBI. The Blast2GO suite (18) was used for this purpose, because it can annotate BLAST results. The homologs for the sequences were retrieved using the QuickGO and InterPro BioMart web services. The **PFAM Domain-based Annotation**—The InterPro and Pfam IDs corresponding to the GO term “ATP binding” (GO ID:0005524) were retrieved using the QuickGO and InterPro BioMart web services. The ATP binding associated domains were queried against the ATP-binding proteome data sets according to spectral quality (high, medium, and low confidence). Their Pfam and InterPro descriptions were identified using the InterProscan web service, which was accessed via the Pipeline Pilot (Accelrys) implementation in the sequence analysis collection. Mapping the domain and the labeled peptide sequences based on the root term “molecular function” and their distance from the root term. The **Gene Ontology Annotation**—The Pfam domains were mapped to Gene Ontology (GO) terms using the lookup table provided by Pfam2go. GO terms are hierarchical and inter-related in nature. All the GO terms originate from three distinct subsumption hierarchy trees, namely cellular component, biological process, and molecular function. Thus, each domain can have multiple GO terms based on the level and type of annotation. An in-house script was written to retrieve GO annotations based on the root term “molecular function” and their distance from the root term.

**Immunoblots**—5 μg of normal and hypoxic lysates were separated on 4–12% BisTris SDS-polyacrylamide gel (Invitrogen). Primary antibodies were either mouse monoclonal (HspX, Ald, Hbha, GlcB, and KatG) or rabbit polyclonal (Rv0569, Rv1738, Rv2626c, Rv2032, and Rv3133c) and diluted to suggested titers. The monoclonal antibody against Hbha was provided as a kind gift from Dr. Mike Brennan (Food and Drug Administration, Silver Spring, MD); the polyclonal antibody against Rv3133 and recombinant protein Rv3133 were provided as kind gifts from Dr. David Sherman (Seattle Biomedical Research Institute, Seattle, WA). The rabbit polyclonal antibodies against Rv0569, Rv1738, Rv2626c, and Rv2032 were made by subcutaneous injection of 0.5 mg of recombinant antigen in an emulsion with Complete Freund’s adjuvant, followed by two additional injections of 0.5 mg of antigen in an emulsion with Incomplete Freund’s adjuvant 21 and 42 days after the initial injection. The recombinant antigens Rv0569 and Rv2626c were made via expression and purification from recombinant clones as described previously (13), and the expression and purification of recombinant Rv1738 and Rv2032 followed methods analogous to that used for recombinant Rv2626c. All antibodies and recombinant clones, with the exception of anti-Rv3133, are available through BEI Resources. Control samples consisted of recombinant proteins generated from *E. coli* or, if unavailable, whole cell lysate from *M. tuberculosis*, H37Rv (Hbha, KatG, Ald). Protein bands were visualized via alkaline phosphatase-conjugated IgG (Sigma-Aldrich). Densitometry analysis was performed via the ImageJ suite (rsbweb.nih.gov).

**RESULTS**

*M. tuberculosis ATPome*—A shotgun proteomics analysis was performed on the enriched subproteome of desthiobiotin-labeled ATP-binding proteins (ATPome). We identified a total of 176 proteins, of which 122 (69%) were labeled via the nucleotide probe, validating the approach for rapid identification of a crucial and potentially druggable subclass of the *M. tuberculosis* proteome (supplemental Table S1). Selective labeling was further validated by ranking the tagged proteins using a metric of ATP labeling based on protein and peptide confidence levels greater than 90% as well as manual interpretation of spectral quality for each peptide sequence labeled with a desthiobiotin tag (19). This ranking accounted for the variation among identified peptides in signal to noise levels and sequence coverage. Proteins were listed by the quality of spectra demonstrating a desthiobiotin-labeled lysine (differential modification of lysine of +196 Da). Low confidence spectra exhibited less than 90% confidence with a minimum of one assigned peptide (number identified was 21; supplemental Tables S1, S2, and S6). Medium confidence peptide spectra had a peptide score between 90 and 95% with two or more unique peptides (number identified was 20; supplemental Tables S1 and S2). Proteins determined to have labeling in the high confidence range exhibited greater than 95% peptide confidence and had two or more unique peptides assigned for identification (number identified was 81; supplemental Tables S1 and S2). A no-probe, streptavidin-only control was performed to account for proteins that have inherent biotin-like domains and may nonspecifically interact with the streptavidin capture resin. Results from the control experiments revealed that only a few proteins, GroEL2 (Rv0440), DnaK (Rv0350), and HspX (Rv2031c), the acyl-carrier protein AcpM (Rv2244), peptidyl-prolyl cis-trans isomerase PpiA (Rv0009), and the naturally biotinylated acetyl carboxylase AccA3 (Rv3285), bound streptavidin nonspecifically in addition to being confidently labeled with the desthiobiotin probe. In the case of GroEL2, DnaK, and HspX, we believe the promiscuous binding to the affinity resin was due to the high abundance of each protein and their chaperoning function. PpiA, although not present in high abundance, also aids in protein folding and thus may associate with the other identified chaperones (20). AccA3 most likely bound the molecular probe (and thus the streptavidin capture resin) by virtue of its affinity for biotin and biotin-like molecules (21). AcpM is functionally associated with AccA3 as both proteins are involved in long chain fatty acid synthesis. Their association in this pathway is visualized via the STRING database (version 9.0 2012 (22)), with curated pathway interactions in the BioCyc version 16.1 pathway collection, and thus may explain the identification of AcpM in this control group. Overall, the utilization of the active site nucleotide probe to capture ATP-binding proteins resulted in
a highly enriched subproteome of essential and potentially druggable targets.

**Functional Annotation of Labeled Proteins**—Over half (59%) of the proteins within the identified *M. tuberculosis* ATPome harbor essential functions to support growth (23, 24), indicating that the *M. tuberculosis* ATPome in general is functionally important. A list of all identified proteins and their annotation as essential or nonessential for in vitro growth is provided in supplemental Table S1. Functional annotation of the proteins was conducted to identify the functional domains and domain families that were selectively labeled and enriched via our chemical proteomic techniques. In total, 218 protein domain families (Pfams) were associated with the GO term ATP binding (GO ID:0005524). The amino acid sequence of each identified protein and covalently labeled peptide sequence was subjected to an InterPro pattern search to identify functional domains and associate these regions within the list 218 annotated domain families (Pfams) (25). It was determined that 13 ATP-associated Pfams were represented in the ATPome dataset across all ranges of labeling confidence (low to high, \( n = 122 \)), and none were represented in proteins identified but not labeled with nucleotide probe (\( n = 54 \)) (supplemental document S1). Among the ATP-associated Pfams were proteins involved with ATP synthesis (PF00006) and peptidoglycan synthesis (Mur Ligase (PF01225)), as well as protein kinases (PF00069). Overall, \( \sim 80\% \) of the ATPome had peptides that could be mapped to Pfam domains (Fig. 1A). The majority of enzyme functions identified were associated with the following activities: small molecule binding (34%), transferase (17%), and oxidoreductase activity (16%) (Fig. 1B).

To further define the functional classes of proteins within the experimental ATPome dataset, a predictive list of potential ATP-binding proteins was generated by query of the search term ATP-binding in The Tuberculosis Database and was combined with a list of proteins from another web-based resource PATRIC (supplemental Table S7) (26, 27). By functional class, categorization of the predicted *M. tuberculosis* ATP-binding proteome revealed that proteins associated with Category 7 (Intermediary Metabolism) and Category 2 (Information Pathways) were equally represented at 20% and that Category 3 (Cell Wall and Processes) represented \( \sim 30\% \) of the predicted subset. When compared with the experimentally derived ATPome subset, 30% of the proteins belong to Category 7, whereas Category 1 (Lipid Metabolism) and Category 10 (Conserved Hypotheticals) represent a collective 33% of the enriched experimental ATPome (Fig. 2). Proteins involved in fatty acid and mycolic acid biosynthesis (Category 1) are of interest due to their key roles in the maintenance of the cell envelope architecture and the essentiality of their encoding genes (24). A complete list of labeled and unlabeled protein IDs and their corresponding functional categories is provided in supplemental Table S1.

**Differential Abundance of Proteins in Normoxic (Normal) Versus Hypoxic State Bacteria**—The strength of this proteomics approach is the ease with which a crucial and druggable slice of the proteome, the ATPome, can be captured and identified over a variety of time points and metabolic states, particularly the so-called “latent” state of *M. tuberculosis* that is associated with drug resistance. Hence, we utilized the active site nucleotide probes to selectively capture and enrich
for the *M. tuberculosis* ATPome under different growth conditions. Normoxic cells growing under standard conditions with aeration and cells grown in limited oxygen conditions (see under “Experimental Procedures”), were harvested, lysed, labeled, and subjected to LC-MS for comparison of their subproteomes (supplemental Table S3). Analysis of NSAF (26, 28) identified 61 differentially abundant proteins based on protein abundance changes that had a *p* value less than 0.05 (19, 29). The log fold change (log FC) values were plotted against the calculated *p* values to visualize the distribution of proteins between the two growth states (Fig. 3). Proteins were determined to be differentially abundant if the calculated log FC was equal to or greater than 1 for proteins with higher abundance in normal samples (Table I) versus negative log FC values for proteins in higher abundance during hypoxic growth (Table II). Patterns of protein function within the captured mycobacterial ATPome demonstrate dynamic changes between normal and hypoxic growth and were seen in the Functional Categories 2, 3, 9, and 10. Categories 0, 1, and 7 remain relatively stable (Fig. 4).

During dormancy and hypoxic growth, *M. tuberculosis* undergoes changes in gene expression that typically involve the up-regulation of enzymes involved in alternative metabolic pathways (i.e. glyoxylate bypass) and those observed to be under the control of the dormancy regulon DosR. The list of proteins in Table II includes the gene products HspX (Rv2031), Acg (Rv2032c), TB31.7 (Rv2623), Rv2624c, and Rv1738. These proteins are directly regulated or co-expressed with the response regulator DosR (Rv3133c) (30).

Isocitrate lyase (icl, Rv0467), the enzyme that catalyzes the reversible cleavage of isocitrate to glyoxylate and succinate (31) and has a role in the growth, survival, and persistence of *M. tuberculosis* in macrophages and mice (32), was found to be labeled with desthiobiotin-ATP on Lys-322 (PFAM, PF00463) and differentially abundant during hypoxic growth. The second enzyme involved in the glyoxylate cycle, malate synthase G (GlcB, Rv1837c), was also labeled with our active site probe; however, in this study its differential abundance in hypoxically grown cultures was not significant (*p* value > 0.60). We did, however, confirm increased protein levels of GlcB via Western blot (Fig. 5 and supplemental Fig. S1). It is well known that the expression of alanine dehydrogenase (*ald*, Rv2780) is also up-regulated during the growth of *M. tuberculosis* under low oxygen conditions (31). It has recently been shown in *M. tuberculosis* and previously in *Mycobacterium smegmatis* that alanine dehydrogenase is responsible for both glycine and alanine dehydrogenase activities (33, 34). The main role of Ald is to generate L-alanine for peptidoglycan and protein synthesis (33). Both Icl and Ald are unique to bacteria and have no human homologs, making them attractive drug targets. Although no inhibitors for Ald have been reported, Icl inhibitors against dormant and logarithmically grown mycobacteria include the 3-nitropropionamides and 5-nitro-2-furroic acid hydrazones (35). Immunoblots of several proteins found to be in higher abundance during hypoxia confirmed the differences in protein levels found via NSAF for differential quantification (Fig. 5 and supplemental Fig. S1).
The mycobacterial serine/threonine kinases (STPK) mediate signal transduction among a variety of intra- and extracellular targets (36, 37). We identified six of the 11 STPK gene products, PknABDHEF. Notably, during normal growth we see an increased abundance in labeled PknD (Rv0931c), PknE (Rv1743), and PknH (Rv1266c). The essential STPK PknA was not found to be differentially abundant between the two growth states (logic $H_1: \text{null}$), whereas PknB was exclusively identified in normally growing cells (data not shown). In addition, several metabolic kinases such as phosphoglycerate kinase Pgk (Rv1743) and the polyphosphate kinase Ppc (Rv2984) were also shown to be more abundant during normal growth (Table I). Aside from PknA and the phosphofructokinase PfkA (Rv3010c) (Table II), the overall lack of protein kinases during dormancy suggests that targeting these proteins under differing metabolic states may not be an efficient means of antimycobacterial killing.

ATP Binding Properties of the M. tuberculosis ATPome—In addition to describing the use of ATP by essential enzymes in the bacterial proteome and identifying those proteins that demonstrated differential abundance patterns between normal and hypoxic states of growth, the third and final goal of this work was to characterize proteins whose ATP-binding function may be utilized in the development of novel ATP-competitive antibiotics. The desthiobiotin labeling of active sites has been used to analyze cellular effects and target selectivity of kinase inhibitors that are clinically approved in the treatment of cancer. In these studies, the binding of the nucleotide probe is quantified in the presence or absence of the drug of choice. These experiments operate under the assumption that for a specific compound-target interaction, the ATP-competitive inhibitor compound will out-compete the binding of the nucleotide probe. As a first step in using this approach to identify native targets of ATP-competitive inhibitors, proteins under both normal oxygen and hypoxic growth conditions were labeled in the presence of excess ATP ($\text{ATP}_{\gamma}\text{S}$). $\text{ATP}_{\gamma}\text{S}$ is a nonhydrolyzable analog of ATP. As the binding of ATP to various protein subunits and active sites can be very dynamic, it has been advantageous to utilize nonhydrolyzable ATP analogs to identify true ATP-binding states of proteins (38). Using this approach, two different sets of proteins were identified as follows: those that bind ATP transiently ($i.e.$ the binding of $\text{ATP}_{\gamma}\text{S}$ and the binding of desthiobiotin-ATP are interchangeable) and proteins for which ATP binding was stable and competitive ($i.e.$ proteins that have a significantly reduced capacity to bind the nucleotide probe in the presence of excess $\text{ATP}_{\gamma}\text{S}$) (supplemental Tables S4 and S5). The relative abundance profiles for these two sets of proteins are exemplified using data from bacilli grown under normal conditions for DevR and ClpC1, respectively (Fig. 6).

Desthiobiotin-labeled peptides were quantified, and those proteins found to have significant fold change differences between samples labeled in the presence or absence of excess $\text{ATP}_{\gamma}\text{S}$ are listed in Tables III (hypoxic) and IV (normoxic). From this analysis, most proteins demonstrated similar ATP binding characteristics (transient or competitive), regardless...
of growth conditions (with more proteins available for comparison when profiled under normal growth). However, a few exceptions exist and are noteworthy. Specifically, Rv0350 (DnaK), Rv0384 (ClpB), and Rv3285 (AccA3) are found in both samples, but they demonstrate a reduced capacity to bind the ATP probe only under hypoxic growth conditions. Similarly, Rv0931 (PknD), Rv2780 (ald), and Rv3596 (ClpC1), are found in both samples, but they demonstrate a reduced capacity to bind the ATP probe only under normal growth conditions. This may be reflective of dynamic binding constants for ATP, based on the availability/loss of co-factors during different growth states. Several attractive drug targets were also identified in this analysis based on their increased abundance during hypoxic growth and their sensitivity to binding the ATP probe in the presence of excess ATP. Specifically, Rv0475 (Hbha), Rv0824 (DesA1), Rv0860 (FadB), Rv1297 (Rho), and Rv2477 represent this phenotype. Of these, Rv2477 is particularly attractive, as it is a macrolide ABC transporter and is associated with increased fluoroquinolone resistance (39). Other proteins, such as Rv0733 (Adk), Rv1310 (AtpD), and Rv3410 (GuaB3), are attractive targets based on the capacity to inhibit their binding and presence in M. tuberculosis regardless of growth state.

**ATP-binding Proteins and Associated Biochemical Pathways**—To find clusters of protein families functionally linked in relevant biochemical pathways, we utilized the list of 81 proteins with increased abundance during normal growth and their hypoxic profiles.

### Table I

| Identified proteins | Accession no. | Molecular mass | Fisher’s exact test (p value) | Normal NSAF | Hypoxic NSAF | Log fold change |
|--------------------|---------------|----------------|-------------------------------|-------------|--------------|----------------|
| Polyphosphate kinase, Ppk | Rv2984 | 83 kDa | (0.0000) | 66 | 1 | 6.04 |
| Phosphoglycerate kinase, Pgk | Rv1437 | 43 kDa | (0.0000) | 57 | 2 | 4.83 |
| Acyl-CoA dehydrogenase, FadE4 | Rv0231 | 63 kDa | (0.0002) | 23 | 1 | 4.52 |
| Dehydrogenase | Rv3389c | 30 kDa | (0.0010) | 19 | 1 | 4.25 |
| 19-kDa lipoprotein antigen precursor, LpqH | Rv3763 | 15 kDa | (0.0021) | 17 | 1 | 4.09 |
| Immunogenic protein, Mpt64 | Rv1980c | 25 kDa | (0.0021) | 17 | 1 | 4.09 |
| Hypothetical protein | Rv2319c | 32 kDa | (0.0021) | 17 | 1 | 4.09 |
| Aminomethyltransferase, GcvT | Rv2211c | 40 kDa | (0.0021) | 17 | 1 | 4.09 |
| Superoxide dismutase soda | Rv3846 | 23 kDa | (0.0031) | 16 | 1 | 4.00 |
| Cysteinyl-tRNA synthetase 1, CysS1 | Rv3580c | 52 kDa | (0.0031) | 16 | 1 | 4.00 |
| DNA polymerase I, PolA | Rv1629 | 98 kDa | (0.0031) | 16 | 1 | 4.00 |
| Transmembrane serine/threonine-protein kinase E, PknE | Rv1743 | 61 kDa | (0.0031) | 16 | 1 | 4.00 |
| Iron-regulated short-chain dehydrogenase/reductase | Rv3224 | 30 kDa | (0.0006) | 26 | 2 | 3.70 |
| Fatty-acid-CoA ligase, FadD23 | Rv3826 | 63 kDa | (0.0099) | 13 | 1 | 3.70 |
| Leucyl-tRNA synthetase, LeuS | Rv0041 | 108 kDa | (0.0150) | 12 | 1 | 3.58 |
| Pyridoxamine 5-phosphate oxidase, PdxH | Rv2607 | 25 kDa | (0.0150) | 12 | 1 | 3.58 |
| Conserved hypothetical protein | Rv2624c | 29 kDa | (0.0002) | 34 | 3 | 3.50 |
| Aldehyde dehydrogenase | Rv0458 | 55 kDa | (0.0066) | 19 | 2 | 3.25 |
| Acetyl-/propionyl-CoA carboxylase α subunit, AccA1 | Rv2501c | 71 kDa | (0.0001) | 45 | 5 | 3.17 |
| Fatty-acid-CoA ligase, FadD7 | Rv0119 | 55 kDa | (0.0020) | 27 | 3 | 3.17 |
| Electron transfer flavoprotein β subunit, FixA | Rv3029c | 28 kDa | (0.0020) | 27 | 3 | 3.17 |
| Fatty-acid-CoA ligase, FadD36 | Rv1193 | 50 kDa | (0.0460) | 9 | 1 | 3.17 |
| Transf erase | Rv1201c | 33 kDa | (0.0460) | 9 | 1 | 3.17 |
| Phosphoribosylamine-glycine ligase, PurD | Rv0772 | 44 kDa | (0.0028) | 26 | 3 | 3.12 |
| Pyruvate kinase, PyKα | Rv1617 | 51 kDa | (0.0021) | 31 | 4 | 2.95 |
| Low molecular weight protein antigen, Cfp2 | Rv2376c | 17 kDa | (0.0250) | 15 | 2 | 2.91 |
| Glutamyl-tRNA synthetase | Rv3009c | 55 kDa | (0.0000) | 83 | 12 | 2.79 |
| Transmembrane serine/threonine-protein kinase H, PknH | Rv1266c | 67 kDa | (0.0036) | 33 | 5 | 2.72 |
| Conserved alanine-rich protein | Rv2744c | 29 kDa | (0.0480) | 13 | 2 | 2.70 |
| Transmembrane serine/threonine-protein kinase D, PknD | Rv0931c | 70 kDa | (0.0005) | 51 | 8 | 2.67 |
| Conserved hypothetical protein | Rv2159c | 36 kDa | (0.0320) | 18 | 3 | 2.58 |
| ATP-dependent protease ATP-binding subunit, CipC1 | Rv3596c | 94 kDa | (0.0000) | 181 | 32 | 2.50 |
| Glutamine synthetase, GlnA2 | Rv2222c | 50 kDa | (0.0280) | 22 | 4 | 2.46 |
| 30 S ribosomal protein S1, RpsA | Rv1630 | 53 kDa | (0.0360) | 21 | 4 | 2.39 |
| Succinyl-CoA synthetase β chain, SucC | Rv0951 | 41 kDa | (0.0170) | 40 | 9 | 2.15 |
| Endopeptidase ATP-binding protein chain B, CipB | Rv0384c | 93 kDa | (0.0003) | 131 | 33 | 1.99 |
| Glutamine synthetase, GlnA1 | Rv2220 | 54 kDa | (0.0200) | 65 | 18 | 1.85 |
| 10-kDa chaperonin, GroES | Rv3418c | 11 kDa | (0.0049) | 136 | 41 | 1.73 |

*a* A value of 1 indicates an NSAF of 0.
fidently labeled proteins and expanded our dataset to include nonlabeled proteins that were confidently identified by mass spectrometry (i.e. proteins with total spectral counts across biological replicates >5 with 90% peptide probability) irrespective of ATP labeling (supplemental Table S1). Functional association networks using the web-based Search Tool for

### Table II

| Identified proteins                                                                 | Accession no. | Molecular mass | Fisher’s exact test (p value) | Normal NSA | Hypoxic NSAF | Log fold change |
|-----------------------------------------------------------------------------------|---------------|----------------|-------------------------------|------------|-------------|-----------------|
| Conserved hypothetical protein                                                    | Rv2623        | 32 kDa         | 0.0000                        | 1          | 14          | -3.81           |
| Acyl-(acyl-carrier protein) desaturase, DesA1                                     | Rv0824c       | 39 kDa         | 0.0000                        | 1          | 12          | -3.58           |
| Hypothetical protein, Acg                                                          | Rv2032        | 37 kDa         | 0.0003                        | 1          | 8           | -3.00           |
| Phosphoribosylaminomiazole-succinocarboxamide synthase, PurC                      | Rv0780        | 33 kDa         | 0.0010                        | 1          | 7           | -2.81           |
| Ketol-acid reductoisomerase, IlvC                                                  | Rv3001c       | 36 kDa         | 0.0010                        | 1          | 7           | -2.81           |
| Acyl-(acyl-carrier protein) desaturase DesA2                                       | Rv1094        | 31 kDa         | 0.0000                        | 2          | 12          | -2.58           |
| Isocitrate lyase, Icl                                                               | Rv0467        | 47 kDa         | 0.0033                        | 1          | 6           | -2.58           |
| 10-kDa culture filtrate antigen, EsxB                                              | Rv3874        | 11 kDa         | 0.0100                        | 1          | 5           | -2.32           |
| 40-kDa secreted l-alanine dehydrogenase, Ald                                       | Rv2780        | 39 kDa         | 0.0000                        | 29         | 134         | -2.21           |
| Citrate synthase I, GltA2                                                          | Rv0896        | 48 kDa         | 0.0320                        | 1          | 4           | -2.00           |
| DNA gyrase subunit B, GyrB                                                         | Rv0005        | 78 kDa         | 0.0320                        | 1          | 4           | -2.00           |
| Transcription termination factor p                                                 | Rv1297        | 65 kDa         | 0.0000                        | 4          | 15          | -1.91           |
| Iron-regulated heparin-binding hemagglutinin, HbhA                                | Rv0475        | 22 kDa         | 0.0004                        | 3          | 11          | -1.87           |
| ATP synthase α chain, AtpA                                                         | Rv1308        | 59 kDa         | 0.0011                        | 4          | 11          | -1.46           |
| Macrolide-transport ATP-binding protein ABC transporter                             | Rv2477c       | 62 kDa         | 0.0005                        | 5          | 13          | -1.38           |
| 6-Phosphofructokinase, PfKα                                                       | Rv3010c       | 37 kDa         | 0.0140                        | 5          | 9           | -0.85           |
| Fatty-acid oxidation protein, FadB                                                  | Rv0860        | 76 kDa         | 0.0010                        | 10         | 17          | -0.77           |
| Heat shock protein, HspX                                                            | Rv2031c       | 16 kDa         | 0.0000                        | 133        | 223         | -0.75           |
| Conserved hypothetical protein                                                     | Rv1738        | 11 kDa         | 0.0019                        | 10         | 16          | -0.68           |
| Bifunctional polyribonucleotide nucleotidytransferase, GpsI                        | Rv2783c       | 80 kDa         | 0.0026                        | 13         | 18          | -0.47           |
| Adenosylhomocysteinase, SahH                                                       | Rv3248c       | 54 kDa         | 0.0000                        | 45         | 53          | -0.24           |
| Iron-regulated aconitate hydratase, Acn                                             | Rv1475c       | 102 kDa        | 0.0490                        | 10         | 11          | -0.14           |

*A value of 1 indicates an NSAF of 0.

**Fig. 4. Comparison of functional categories.** Desthiobiotin-labeled proteins found to be differentially abundant between normal and hypoxic growth were sorted based on functional category and compared with proteins in each category predicted to be ATP binding. Categories 4 (Stable RNAs), 5 (Insertion Sequences and Phages), and 6 (PE/PPE) were not represented in the experimental ATPome dataset.
were generated from the 81 ATP-binding proteins combined with the 54 unlabeled proteins. Emerging from this data set we visualized clusters of associated protein families (Fig. 7), including members of the polyketide synthase family (Category 1, Lipid Metabolism), ribosomal protein synthesis (Category 2, Information Pathways), and mycolic acid and peptidoglycan synthesis (Category 3, Cell Wall and Processes). Polyketide synthases are large multidomain proteins involved in lipid and mycolic acid biosynthesis. Pks5, Pks12, and Pks13 as well as the phthiocerol dimycoserate synthases PpsABCDE and mycocerosic acid synthase (Mas) work in

Fig. 5. Densitometry analysis. Several proteins found to be differentially abundant by NSAF were probed for immunoreactivity via Western blot. The densitometry analysis of these blots corroborates the differences in protein levels between Normal and Hypoxic growth (left). The densitometry of each protein with its corresponding positive control (recombinant protein) is reported (upper). All immunoblot images are provided in supplemental Fig. S1.

Fig. 6. NSAF profiles of ATP-labeled peptides in the presence/absence of excess ATPγS. Transient binding of ATP was observed in many proteins, including the DNA-binding transcriptional regulator DevR (left). In the presence of excess ATP (ATPγS), the spectral count profiles of the ATP-binding subunit of ClpC1 were significantly reduced (right) in cultures grown under normal conditions.
coordination to synthesize the cell wall-associated and virulence determinant phthiocerol dimycocerosate (40, 41). Within this group of proteins, PpsC was found to bind ATP. PpsC catalyzes the complete reduction of malonyl-CoA in the synthesis of phthiocerol. The localization of the ATP nucleotide probe was not within any of the annotated domains of PpsC.

| Competitive binders of desthiobiotin-ATP | Gene no. | Molecular mass | p value | Avg. NSAF_ATPγSα | Avg. NSAF_ATPβ | -Fold change |
|------------------------------------------|----------|----------------|---------|------------------|----------------|-------------|
| Fatty-acid oxidation protein, FadB        | Rv0860   | 76 kDa         | 0.0039  | 1                | 6.33           | 6.33        |
| Inosine-5-monophosphate dehydrogenase, GuaB3 | Rv3410c  | 39 kDa         | 0.0056  | 1                | 6.00           | 6.00        |
| Transcription termination factor ρ        | Rv1297   | 65 kDa         | 0.0079  | 1                | 5.67           | 5.67        |
| Macrolide-transport ATP-binding protein, ABC transporter | Rv2477c  | 62 kDa         | 0.0160  | 1                | 5.00           | 5.00        |
| Endopeptidase ATP-binding protein chain B, ClpB | Rv0384c  | 93 kDa         | 0.0031  | 2.5              | 11.67          | 4.67        |
| Iron-regulated heparin-binding hemagglutinin, HbhA | Rv0475  | 22 kDa         | 0.0320  | 1                | 4.33           | 4.33        |
| Adenosylhomocysteinase, SahH               | Rv3248c  | 54 kDa         | 0.0065  | 5.5              | 18.33          | 3.33        |
| Chaperone protein, DnaK                    | Rv0350   | 67 kDa         | 0.0001  | 16               | 50.00          | 3.13        |
| Adenylate kinase, Adk                      | Rv0733   | 20 kDa         | 0.0400  | 8                | 20.33          | 2.54        |
| Acyl-(acyl-carrier protein) desaturase, DesA1c | Rv0824c  | 39 kDa         | 0.2200  | 2                | 4.67           | 2.33        |
| ATP synthase β chain, AtpDc                | Rv1310   | 53 kDa         | 0.1500  | 5                | 11.33          | 2.27        |
| Transient binders of desthiobiotin-ATP    |          |                |         |                  |                |             |
| 50 S ribosomal protein L7/L12, RplL        | Rv0652   | 13 kDa         | 0.3100  | 4.5              | 8.67           | 1.93        |
| 10-kDa chaperonin, GroES                   | Rv3418c  | 11 kDa         | 0.3600  | 8                | 14.33          | 1.79        |
| ATP-dependent protease ATP-binding subunit, ClpC1 | Rv3596c  | 94 kDa         | 0.5000  | 7                | 11.33          | 1.62        |
| 60-kDa chaperonin 1, GroEL1                | Rv3417c  | 56 kDa         | 0.5100  | 3.5              | 5.67           | 1.62        |
| Conserved hypothetical protein             | Rv2623   | 32 kDa         | 0.5600  | 3.5              | 5.33           | 1.52        |
| 40-kDa secreted l-alanine dehydrogenase, Ald | Rv2780  | 39 kDa         | 0.0520  | 36               | 45.33          | 1.26        |
| Cold shock protein A, CspA                 | Rv3648c  | 7 kDa          | 0.4400  | 4                | 5.00           | 1.25        |
| 60-kDa chaperonin 2, GroEL2                | Rv0440   | 57 kDa         | 0.0000  | 135              | 168.67         | 1.25        |
| Heat shock protein, HspX                   | Rv2031c  | 16 kDa         | 0.0002  | 67.5             | 75.00          | 1.11        |
| Transmembrane serine/threonine-protein kinase A, PknA | Rv0015c | 46 kDa         | 0.3100  | 4                | 4.33           | 1.08        |
| Isocitrate lyase, Icl                      | Rv0467   | 47 kDa         | 0.4300  | 2.5              | 2.67           | 1.07        |
| Integration host factor, MihF               | Rv1388   | 21 kDa         | 0.0280  | 19               | 20.00          | 1.05        |
| 6-Phosphofructokinase, PfkA                | Rv3010c  | 37 kDa         | 0.3200  | 3.5              | 3.67           | 1.05        |
| Bifunctional polyribonucleotide nucleotidyltransferase, Gpsl | Rv2783c | 80 kDa         | 0.1600  | 6.5              | 6.67           | 1.03        |
| Iron-regulated elongation factor tu tuf    | Rv0685   | 44 kDa         | 0.0120  | 20.5             | 20.33          | 0.99        |
| Propionyl-CoA carboxylase β chain 5, AccD5 | Rv3280   | 59 kDa         | 0.5000  | 1.5              | 1.33           | 0.89        |
| Conserved hypothetical protein             | Rv3269   | 10 kDa         | 0.1600  | 4.5              | 4.00           | 0.89        |
| Electron transfer flavoprotein β subunit, FixA | Rv3029c | 28 kDa         | 0.3300  | 2                | 1.67           | 0.83        |
| Conserved hypothetical protein             | Rv1738   | 11 kDa         | 0.0390  | 7.5              | 6.00           | 0.80        |
| Iron-regulated conserved hypothetical protein | Rv1636  | 15 kDa         | 0.0093  | 10               | 7.33           | 0.73        |
| Transmembrane serine/threonine-protein kinase D, PknD | Rv0931c | 70 kDa         | 0.0100  | 6                | 3.33           | 0.56        |
| Conserved hypothetical protein             | Rv3127   | 39 kDa         | 0.0640  | 3                | 1.67           | 0.56        |

α Average spectral count of desthiobiotin-labeled peptides in the presence of 500 μM ATPγS. A value of 1 indicates 0 spectral counts in the presence of excess ATP analog.

β Average spectral count of desthiobiotin-labeled peptides with no excess ATPγS.

p value of >0.05.
## Competitive and transient binders of desthiobiotin-ATP in normal cultures

| Genomic No. | Molecular Mass | p Value | Avg. NSAF-ATP<sub>a</sub> | Avg. NSAF-ATP<sub>b</sub> | Fold Change |
|-------------|----------------|---------|---------------------------|---------------------------|-------------|
| **Competitive Binders of Desthiobiotin-ATP** | | | | | |
| Acetylpropanoyl-CoA carboxylase subunit, AccA | 71 kDa | (0.0000) | 1.00 | 15.67 | 15.67 |
| Glutamyl-tRNA synthetase subunit B, GatB | 55 kDa | (0.0000) | 2.33 | 28.33 | 12.14 |
| Inosine-5-monophosphate dehydrogenase, GuaB | 39 kDa | (0.0000) | 1.00 | 11.67 | 11.67 |
| Transmembrane serine/threonine-protein kinase H, PknH | 67 kDa | (0.0000) | 1.00 | 11.33 | 11.33 |
| Fatty-acid-CoA ligase, FadD7 | 55 kDa | (0.0000) | 1.00 | 9.67 | 9.67 |
| Phosphoribosylamine-glycine ligase, PurD | 44 kDa | (0.0000) | 1.00 | 9.33 | 9.33 |
| Tryptophanyl-tRNA synthetase, TrpS | 36 kDa | (0.0001) | 1.00 | 7.00 | 7.00 |
| Polyphosphate kinase, Ppk | 83 kDa | (0.0000) | 3.33 | 22.67 | 6.80 |
| Phosphoglycerate kinase, Pgk | 63 kDa | (0.0014) | 1.00 | 5.00 | 5.00 |
| Cysteinyl-tRNA synthetase 1, CysS1 | 52 kDa | (0.0005) | 1.00 | 7.00 | 7.00 |
| Fatty-acid-CoA ligase, FadD23 | 57 kDa | (0.0000) | 1.00 | 4.77 | 4.77 |
| ATP-dependent protease ATP-binding subunit, ClpC1 | 52 kDa | (0.0000) | 1.00 | 4.00 | 4.00 |
| Anchored-membrane serine/threonine-protein kinase, PknF | 51 kDa | (0.0130) | 1.00 | 3.67 | 3.67 |
| Chaperone protein, HtpG | 73 kDa | (0.0130) | 1.00 | 3.67 | 3.67 |
| DNA polymerase I, PolA | 98 kDa | (0.0078) | 1.67 | 6.00 | 3.60 |
| Acetate kinase, AckA | 41 kDa | (0.0220) | 1.00 | 3.33 | 3.33 |
| UDP-N-acetylmuramoyl-d-alanyl-d-alanine ligase, MurF | 52 kDa | (0.0220) | 1.00 | 3.33 | 3.33 |
| Conserved hypothetical protein | 23 kDa | (0.0220) | 1.00 | 3.33 | 3.33 |
| Fatty-acid-CoA ligase, FadD28 | 63 kDa | (0.0077) | 2.33 | 7.67 | 3.29 |
| Nucleoside diphosphate kinase, NdkA | 15 kDa | (0.0066) | 3.00 | 9.33 | 3.11 |
| 40-kDa secreted L-alanine dehydrogenase, Ald | 39 kDa | (0.0017) | 5.00 | 15.00 | 3.00 |
| Adenosylhomocysteinase, SahH | 54 kDa | (0.0017) | 5.00 | 15.00 | 3.00 |
| Pyruvate kinase, PykA | 51 kDa | (0.0053) | 3.67 | 11.00 | 3.00 |
| Malate synthase G, GlcB | 80 kDa | (0.0420) | 1.67 | 6.00 | 3.60 |
| Aldehyde dehydrogenase | 55 kDa | (0.0350) | 2.33 | 7.00 | 2.63 |
| Adenylate kinase, Adk | 20 kDa | (0.0035) | 17.33 | 36.67 | 2.12 |
| transmembrane serine/threonine-protein kinase D, PknD | 70 kDa | (0.0400) | 8.67 | 17.67 | 2.04 |
| ATP synthase beta chain, AtpD | 53 kDa | (0.0250) | 13.33 | 26.33 | 1.98 |

### Transient binders of desthiobiotin-ATP

| Genomic No. | Molecular Mass | p Value | Avg. NSAF-ATP<sub>a</sub> | Avg. NSAF-ATP<sub>b</sub> | Fold Change |
|-------------|----------------|---------|---------------------------|---------------------------|-------------|
| Endopeptidase ATP-binding protein chain B, ClpB | 93 kDa | (0.0410) | 25.33 | 44.33 | 1.75 |
| 3-Hydroxyacyl-thioester dehydrogenase, HtdY | 30 kDa | (0.4000) | 4.67 | 7.00 | 1.50 |
| Iron-regulated short-chain dehydrogenase/reductase | 30 kDa | (0.4100) | 6.33 | 9.33 | 1.47 |
| Aminomethyltransferase, GcvT | 40 kDa | (0.4300) | 4.33 | 6.33 | 1.43 |
| Phosphopantetheine adenylyltransferase, KdtB | 18 kDa | (0.4700) | 2.33 | 3.33 | 1.43 |
| Chaperone protein, DnaK | 67 kDa | (0.3800) | 40.00 | 57.00 | 1.43 |
| Cold shock protein A, CspA | 7 kDa | (0.5300) | 5.67 | 7.67 | 1.35 |
| Secreted fibronectin-binding protein antigen | 53 kDa | (0.5400) | 2.00 | 2.67 | 1.33 |
| Iron-regulated peptidyl-prolyl cis-trans isomerase, PpiA | 9 kDa | (0.5400) | 3.00 | 4.00 | 1.33 |
| Glutamine synthetase, GlnA2 | 50 kDa | (0.5500) | 6.00 | 8.00 | 1.33 |
| Pyruvate dehydrogenase E2 component, SucB | 57 kDa | (0.5500) | 7.33 | 9.67 | 1.32 |
| Acyl-CoA dehydrogenase, FadE5 | 66 kDa | (0.5700) | 3.33 | 4.33 | 1.30 |
| Pyruvate dehydrogenase E1 component, AceE | 100 kDa | (0.5700) | 4.67 | 6.00 | 1.29 |
| 60-kDa chaperonin 2, GroEL2 | 57 kDa | (0.0180) | 279.00 | 352.00 | 1.26 |
| Iron-regulated conserved hypothetical protein | 15 kDa | (0.3500) | 4.67 | 6.00 | 1.29 |
| Propionyl-CoA carboxylase beta chain 5, AccD5 | 59 kDa | (0.5800) | 3.00 | 3.67 | 1.22 |
| Low molecular weight protein antigen, Clp2 | 17 kDa | (0.5000) | 4.67 | 5.67 | 1.21 |
| Glutamine synthetase, GlnA1 | 54 kDa | (0.3000) | 17.67 | 21.33 | 1.21 |
| Heat shock protein, HspX | 16 kDa | (0.1800) | 36.00 | 43.33 | 1.20 |
Although PpsC is a nonessential enzyme, its associated protein partners identified in this study do play essential roles (pk12/13) (41).
FIG. 7. Protein-protein interaction networks of the *M. tuberculosis* ATPome. The list of protein IDs from our MS analysis was input into the STRING database (STRING version 9.0) to identify known and predicted functional networks. 48% of the proteins in our shotgun analysis were shown to be functionally associated with at least one known interacting partner. Emerging protein clusters are functionally relevant in basic metabolism (*i.e.* respiratory chain and protein synthesis), cell wall biosynthesis (*i.e.* fatty acid and peptidoglycan synthesis), and virulence (*i.e.* lipid synthesis).
were found to be differentially abundant between normal and hypoxic states. We identified 16 gene products involved in the synthesis of proteins, 14 of which are essential.

Proteins involved in fatty acid and mycolic acid biosynthesis found within our mycobacterial ATPome are listed in supplemental Table S1. Of note, the essential acyl (ACP) membrane-bound desaturases, DesA1 and DesA2, catalyze the introduction of the first double bond in saturated C16 and C18 fatty acids. desA1 and desA2 are essential genes for mycobacterial survival, and DesA1 is predicted to be a relevant drug target based on interactome and genome-scale structural analysis (42). Both enzymes were found to be in higher abundance during hypoxia with a log FC of 12 and 6, respectively (Table I). A third member of this family DesA3 is a putative target of the thiourea drug isoxyl (43), but it was not identified in our study. The M. tuberculosis genome contains three biotin-dependent essential acyl-CoA carboxylases (AccA1–3). Although the biotin binding domain of these enzymes did allow for the nonspecific attachment to the streptavidin capture affinity resin (as discussed above), their ATP-binding function is essential to their enzymatic activity, and labeling with nucleotide probe was located within an annotated nucleotide binding domain (Lys-116) (44).

Finally, we identified the d-glutamic acid ligase (MurD), the meso-diaminopimelic acid ligase (MurE), and the dipeptide d-Ala–d-Ala ligase (MurF) in our ATPome dataset. Interestingly, we also identified the dihydrolipicolate reductase (DapB). Although primarily associated with processes of intermediary metabolism due to its function in the biosynthesis of L-lysine, it is also involved in the synthesis of meso-diaminopimelic acid, an amino acid contained in the core tetrapeptide of peptidoglycan (45). An M. tuberculosis mutant lacking functional DapB has been classified as a slow growth mutant (46), and this protein may represent a uniquely lethal drug target as this metabolic function is unique to prokaryotes.

**DISCUSSION**

The results represented in this study are among the first to describe the ATP-binding proteome of the pathogenic organism, M. tuberculosis and present a relevant proteomic comparison between normally growing and hypoxic state bacteria. The majority of these proteins are essential gene products and may be relevant therapeutic targets. We quantitatively measured the differences in protein levels between normally growing and hypoxic state bacteria and provided preliminary data into the binding characteristics and utilization of ATP across multiple classes of functional enzymes. Using desthiobiotin nucleotide probes in competition with ATP analogs provides the framework necessary to pursue antimicrobial inhibitors whose mode of action relies on competition within the ATP-binding site of select protein targets (4, 47). The utilization of ATP in lipid and cell wall biosynthesis pathways makes it tempting to speculate that a selective and broad-spectrum nucleotide-competitive compound may affect these critical processes in such a way as to alter cell wall architecture and integrity. Because the cell wall of M. tuberculosis is a potent barrier against small molecule therapeutics, agents that alter the cell wall have been shown to increase drug sensitivity and help circumvent the problems of multidrug and extensively drug-resistant bacteria (48).

Beyond the identification of these proteins as targets for small molecule inhibitors, the ATP-binding proteins of M. tuberculosis comprise a very unique and functional subset of the M. tuberculosis proteome. The distribution of ATP-binding proteins among a variety of functional classes supports the general hypothesis that proteins of the mycobacterial ATPome provide necessary mechanisms of adaptation utilized in the maintenance of growth under a variety of microenvironmental conditions. Applying functional categories across a predicted subset of ATP-binding proteins demonstrated several features of note: 1) The predicted ATP-binding protein distribution by function more closely reflects experimentally identified proteins grown under normal conditions, 2) Lipid biosynthesis and intermediary metabolism are overly represented experimentally, and compared with predicted annotations, a trend recently seen elsewhere (26), 3) Predictions for cell wall and processes were under-represented experimentally compared with predicted subsets. This is most likely due to our experimental approaches, and further investigation of the ATP-binding properties of cell wall proteins may lead to better experimental representation within this category. The functional Category 10, Conserved Hypotheticals, represents proteins whose function remains uncharacterized and accounts for approximately one-quarter of the M. tuberculosis proteome. Recent re-annotation and prediction efforts concluded that the majority of hypothetical proteins could be redistributed among the categories of Small Molecule Metabolism, Cell Wall Processes, and Lipid Metabolism (49, 50). The utilization of ATP by these hypothetical proteins may provide further insight into their cellular roles and enzymatic functions and further lead to key insights in the study of M. tuberculosis pathogenicity. The idea of ATP binding and hydrolysis acting as a molecular switch controlling the transition into hypoxia has been observed in the study of mammalian models of low-oxygen conditions (51, 52). For mycobacteria, one class of ATP-binding proteins, the Universal Stress Proteins (USPs), may be involved in the responses to changes in environmental and nutrient conditions leading to variations in virulence and adaptation. The USPs identified in our study include Rv2005c, Rv2028c, Rv2319c, Rv2623, Rv2624, and Rv2626c. Several years ago, Drumm and Chan et al. (53), investigated the nucleotide binding capabilities of Rv2623 and its role as an USP. Gene deletion mutants in M. tuberculosis demonstrated a hypervirulent phenotype that failed to enter into dormancy within susceptible Hartley guinea pigs. Disruption of the ATP-binding site of Rv2623 resulted in similar attenuated phenotypes described for the deletion mutants. It was hypothesized that the binding of ATP by similar USPs could be a regulatory
mechanism utilized in the transition from normal growth to an oxygen-poor state of dormancy.

In our profile of ATP-binding proteins from normally and hypoxically grown \( M.\) \textit{tuberculosis}, we identified several proteins known to be under the control of the dormancy regulon \( \text{dosR} \). The DevR-DevS two component system (TCS) is implicated in virulence and mediates the expression of \( \sim 48 \) dormancy-associated genes when \( M.\) \textit{tuberculosis} adapts to hypoxia and is exposed to other stress factors like nitric oxide, carbon monoxide, and ascorbic acid (54). The \( \sim 48 \) genes that comprise the \( \text{devR} \) dormancy regulon include well known genes like \( \text{hspX} \) (the \( \alpha \)-crystallin like chaperone), the nitrate reductase \( \text{acg} \), and several uncharacterized hypothetical proteins such as Rv0569, Rv1738, and Rv2626c. A derivative of phenylcoumarin reduced the survival of hypoxically adapted \( M.\) \textit{tuberculosis} and also inhibited DevR binding to target DNA (55). We would expect to have identified the sensor kinase DevS; however, it did not meet criteria for final inclusion, most likely due to its subcellular location with the plasma membrane. The response regulator DevR/\text{DosR} (Rv3133c) was likely due to its subcellular location with the plasma membrane. The response regulator DevR/\text{DosR} (Rv3133c) was shown to be confidently labeled with the ATP probe at the C-terminal DNA binding domain. In our competition experiments with 100-fold excess ATP-S, the desthiobiotin nucleotide probe was still able to bind and label two lysines (Lys-179 and Lys-182) within the helix-turn-helix DNA binding domain of DevR (UniProt ID >sp|P95193|167–186). The physiological implications of nucleotide binding to DevR remain to be elucidated, although Ansong \textit{et al.} (26), also observed this phenomena for DNA-binding proteins. Genomic scale surveys of essential genes of \( M.\) \textit{tuberculosis} and transcriptome-wide analyses of the bacterial response to environmental or metabolic conditions mimicking the host environment have been carried out in the pursuit of attractive, novel, and functionally relevant drug targets (30, 56, 57). Additionally, large scale proteomic profiling under simulated \textit{in vitro} conditions or \textit{in vivo} have also been performed (58–60). However, models of gene regulation, protein-protein interactions, and unique metabolic pathways at the systems level remains incomplete, especially those designed to characterize functional changes that mediate the switch into dormancy and thus may be key therapeutic targets for latent tuberculosis (61, 62). Through the study of the \( M.\) \textit{tuberculosis} ATPome, we have defined a functionally linked analysis among essential gene products of the mycobacterial proteome. Furthermore, the chemoproteomic technique employed in these studies may be used to broaden the functional annotations and physiological roles of many of these nucleotide-binding proteins, especially in response to differing metabolic conditions. For drug discovery efforts, this work supports a growing body of evidence regarding the potential of pursuing antimicrobial inhibitors whose mode of action relies on competition within the ATP-binding site of select proteins (5, 47, 63–65). Future studies focused on measuring the abundance levels of promising inhibitor targets throughout the course of infection in \( M.\) \textit{tuberculosis}, as well as similar studies in other biologically important pathogens that persist in multiple growth and metabolic states, will further demonstrate the broad applicability of this technique in drug discovery programs.

\textbf{Acknowledgments}—We thank Phillip Knabenbauer for assistance with the growth and manipulation of \( M.\) \textit{tuberculosis} and Tige Rustad at the Seattle Biomedical Research Institute for providing information regarding growth of \( M.\) \textit{tuberculosis} under hypoxia.

* This work was supported, in whole or in part, by National Institutes of Health Grant HHSN266200400091c from NIAID (to K.M.D.). This work was also supported by administrative funds by the Colorado State University Department of Microbiology, Immunology, and Pathology (to K.M.D.).

\[ \text{S} \] This article contains supplemental material.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomeexchange.org) via the PRIDE partner repository (16) with the dataset identifier PXD000141.

\[ \text{S} \] Supported by a postdoctoral fellowship grant from Indo-US Science and Technology Forum.

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