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Identification of Fur, Aconitase, and Other Proteins Expressed by *Mycobacterium tuberculosis* under Conditions of Low and High Concentrations of Iron by Combined Two-Dimensional Gel Electrophoresis and Mass Spectrometry

DIANE K. WONG,[1] BAI-YU LEE,[2] MARCUS A. HORWITZ,[2] AND BRADFORD W. GIBSON[1]*

Department of Chemistry and Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0446,[1] *and Department of Medicine, School of Medicine, University of California, Los Angeles, California 90095*[2]

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Iron plays a critical role in the pathophysiology of *Mycobacterium tuberculosis*. To gain a better understanding of iron regulation by this organism, we have used two-dimensional (2-D) gel electrophoresis, mass spectrometry, and database searching to study protein expression in *M. tuberculosis* under conditions of high and low iron concentration. Proteins in cellular extracts from *M. tuberculosis* Erdman strain grown under low-iron (1 μM) and high-iron (70 μM) conditions were separated by 2-D polyacrylamide gel electrophoresis, which allowed high-resolution separation of several hundred proteins, as visualized by Coomassie staining. The expression of at least 15 proteins was induced, and the expression of at least 12 proteins was decreased under low-iron conditions. In-gel trypsin digestion was performed on these differentially expressed proteins, and the digestion mixtures were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry to determine the molecular masses of the resulting tryptic peptides. Partial sequence data on some of the peptides were obtained by using after source decay and/or collision-induced dissociation. The fragmentation data were used to search computerized peptide mass and protein sequence databases for known proteins. Ten iron-regulated proteins were identified, including Fur and aconitase proteins, both of which are known to be regulated by iron in other bacterial systems. Our study shows that, where large protein sequence databases are available from genomic studies, the combined use of 2-D gel electrophoresis, mass spectrometry, and database searching to analyze proteins expressed under defined environmental conditions is a powerful tool for identifying expressed proteins and their physiologic relevance.

The *Mycobacterium tuberculosis* genome sequencing project has provided information on sequences of hundreds of newly identified proteins encoded by this pathogen's DNA. The availability of this information provides new opportunities for increasing our understanding of the pathophysiology of *M. tuberculosis* in the human host. Toward this end, a major next step is to determine the functions of the proteins revealed by the genome project and their interplay under different physiological conditions in the host.

One physiological condition in the host known to be important in *M. tuberculosis* infection is the concentration of iron. Iron is an essential nutrient for all pathogens, but this element appears to play an especially critical role in the pathogenesis of tuberculosis. For example, serum containing poorly saturated transferrin, such as human serum, is tuberculostatic, an effect neutralized by the addition of iron (28, 29).

The amount of iron is severely limited in the host at sites of *M. tuberculosis* replication. A facultative intracellular parasite, *M. tuberculosis* multiplies within macrophages in the lung and elsewhere. Within the macrophage, iron is limited as a result of the effects of the immunomodulator interferon gamma. This cytokine depletes iron in the labile iron pool of the cell by downregulating transferrin receptor expression and the intracellular concentration of ferritin (6, 7). *M. tuberculosis* also multiples extracellularly in lung cavities. In the extracellular space, iron is severely limited owing to the high affinity with which it is bound by the host iron-binding proteins transferrin and lactoferrin.

One measure of the importance of iron to *M. tuberculosis* is the degree to which it goes to obtain this element. The pathogen is known to produce in great abundance at least two high-affinity iron siderophores—exochelins and mycobactins (19, 39, 57). Both exochelins and mycobactins are low-molecular-weight (MW) compounds (MW, ~700 to 1,000) that are nonribosomally synthesized and contain two fatty acid moieties, salicylic acid, and three modified amino acids per molecule. Exochelins are released extracellularly and may be the most abundant molecule exported by *M. tuberculosis*. On a molar basis, the concentration of exochelins in *M. tuberculosis* culture filtrates (~5 μM) (19) is 150-fold that of the 30-kDa (antigen 85b) major secretory protein of *M. tuberculosis* (~30 nM) (22), the most abundant protein exported by this organism. Mycobactins are water-insoluble molecules located in the cell wall of *M. tuberculosis* cells. In previous studies in our laboratories, we characterized *M. tuberculosis* exochelins and found that their core structure resembles that of the mycobactins (19). A shorter alkyl side chain on exochelins and a terminal methyl ester or carboxylic acid moiety on this side chain renders exochelins more polar than mycobactins and hence water soluble. It has been proposed by Macham and colleagues that exochelins bind iron in the aqueous extracellular milieu of the mycobacterium and transfer it to mycobactins in the cell wall for subsequent internalization into the bacterial cytoplasm (39, 62). Consistent with this hypothesis, we have demonstrated that exochelins remove iron from human transferrin

* Corresponding author. Mailing address: School of Pharmacy S-926, 531 Parnassus Ave., University of California, San Francisco, CA 94143-0446. Phone: (415) 476-5320. Fax: (415) 476-0688. E-mail: gibson@socrates.ucsf.edu.

and lactoferrin and transfer it to mycobactins in the cell wall of live *M. tuberculosis* cells (18).

To learn more about the role of iron in the physiology of *M. tuberculosis*, we have been investigating iron-regulated proteins of *M. tuberculosis*. In this study, we have taken advantage of three major scientific or technological advances to gain a more complete picture of how *M. tuberculosis* responds to change in the iron concentration in its environment. The first advance, already noted, is the database generated by the *M. tuberculosis* genome sequencing project (49). The second advance is the development of high-resolution two-dimensional (2-D) gel electrophoresis allowing greatly enhanced separation of proteins. The third advance is the development of mass spectrometric methods for the low-level detection and identification of proteins and peptides. In an effort to learn about cellular components affected by iron levels, we used these three modalities—2-D gel electrophoresis, mass spectrometry (MS), and database searching—to identify proteins expressed under conditions of low or high concentrations of iron. We report the identification of 10 proteins from 11 different gel spots whose expression levels were markedly affected by low- or high-iron concentration conditions. These include two proteins already known to be affected by environmental iron levels in other bacteria, Fur and aconitase, as well as several mycobacterial antigens and enzymes not previously known to be affected by environmental iron levels.

MATERIALS AND METHODS

Bacteria. *M. tuberculosis* Erdman strain (ATCC 35801) was obtained from the lungs of guinea pigs infected with the bacteria by aerosol. Frozen bacterial stocks for use in iron studies were prepared from 7H11 agar plates as described (24).

Medium. Iron-deficient Sauton's broth was prepared by subjecting the broth to a chelating resin as described (8). Briefly, 5 g of Chelex 100 resin (Bio-Rad, Hercules, Calif.) per liter was added to Sauton's medium prepared without ferric ammonium citrate and magnesium sulfate (14), and the medium was stirred at 4°C overnight. The Chelex resin-treated medium was passed through a 0.2-μm-pore-size filter into an acid-washed glass flask. Magnesium sulfate (250 mg/liter) and trace amounts of metals including zinc (2 mg of $ZnSO_4 \cdot 7H_2O$ per liter) and copper (0.5 mg of $CuSO_4$ per liter) were added. This iron-deficient medium was then supplemented with ferric ammonium citrate to the desired iron concentration (1, 15, or 70 μM). The iron concentration in the medium was routinely checked by the ferrozine assay (16).

Cultures. Bacteria from frozen stocks were cultured on 7H11 agar plates at 37°C in 5% CO_2 for 3 weeks, inoculated into Sauton's medium containing 15 μM Fe at an initial optical density at 540 nm (OD_{540}) of 0.05, and cultured for 3 weeks at 37°C in 5% CO_2 without shaking to a final OD_{540} of approximately 1.0. The bacteria were then subcultured into Sauton's medium containing either 1 or 70 μM Fe at an initial OD_{540} of 0.05, grown for 3 weeks to an OD_{540} of 0.7 to 1.0, harvested, and stored at −20°C until use.

Sample preparation and 2-D gel electrophoresis. Approximately 20 ml (wet volume) of bacteria was suspended in 80 ml of phosphate buffer containing 100 μM phenylmethylsulfonyl fluoride, 100 μM benzamidine, and 0.5 mM EDTA. The bacterial suspension was sonicated four times for 10 min each time on an ice bath with a 1-cm-diameter probe attached to a sonicator set at 50% duty cycle and a strength of 5 with pulsing (model W-375; Heat System-Ultrasonics, Inc., Farmingdale, N.Y.) and centrifuged at 10,000 × *g* for 30 min to pellet unbroken bacteria and bacterial cell walls. The supernatant was passed sequentially through a 0.45-μm-pore-size filter and a 0.2-μm-pore-size filter, further clarified by centrifugation at 40,000 × *g* for 2 h, and fractionated by ammonium sulfate precipitation. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Two-dimensional gel electrophoresis was performed as described previously (33) with modifications. Protein samples of 300 μg each were dissolved in sample buffer containing 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 10% glycerol and heated at 95°C for 5 min. The samples were centrifuged at 100,000 × *g* for 10 min to remove any insoluble material and loaded onto 2.4-mm (internal diameter) by 16-cm (length) isoelectric focusing tube gels with a ratio of ampholytes (pH 3 to 10/pH 5 to 7) of 1:4. The samples were focused at 200 V for 2 h, 500 V for 2 h, and 800 V for 16 h. The second-dimension gels were 10 to 20% polyacrylamide linear gradient gels (size, 20 cm by 16 cm by 1.5 mm).

In-gel digestion with trypsin. The in-gel digestion procedure was similar to the methods of Rosenfeld et al. (53) and Sheer (54) with modifications as described below (11a). Protein spots of interest were cut out of the gel and diced into small pieces with a stainless-steel scalpel or a vortex mixer and placed in siliconized microcentrifuge tubes. The gel was destained and dehydrated by washing three times (~10 min) with 25 mM NH_4HCO_3–50% acetonitrile (or until the Coomassie stain was no longer visually detectable). The destained gel particles were then dried under vacuum for 30 min. After rehydration of the particles with a minimal amount of 25 mM NH_4HCO_3 with 0.1 μg of trypsin per μl, the protein was digested overnight at 37°C. Recovery of the peptides was accomplished by extracting the digestion mixture three times with 50% acetonitrile–5% trifluoroacetic acid. In an effort to reduce the amount of volatile salts (e.g., trifluoroacetic acid and NH_4HCO_3), the recovered peptides were concentrated in a Speed-Vac vacuum centrifuge (to a final volume of ~5 μl) and rehydrated at least three times. Control digestions were performed on gel slices that did not contain any protein and revealed trypsin autoproteolysis products and keratin contaminants that were readily identified in the subsequent mass spectrometric analyses (see below).

MALDI-TOF MS of the unseparated digests. As described in Matsui et al. (41), portions (approximately 1/10th) of the unseparated tryptic digestion mixture were mixed at a 1:1 (vol/vol) ratio with an α-cyano-4-hydroxycinnamic acid matrix (Hewlett-Packard) and analyzed on a ToFSpec SE matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Micromass Inc., Manchester, United Kingdom) with a nitrogen laser, operated in reflectron mode (25). A standard peptide mixture was used to externally calibrate all mass spectra. Postsource decay (PSD) sequencing (26) involved gating a precursor ion to selectively transmit an individual peptide and its metastable fragment ions to the reflectron. The PSD experiments were carried out by varying the reflectron voltage in 9 to 11 steps, with the voltage at each step being reduced to 75% of that at the previous step. The complete PSD spectrum was produced by stitching the segments from individual steps together. Calibration in PSD mode was done by using the fragment ions from a standard peptide, adrenocorticotropic hormone 18-39.

MALDI-CID MS. Small aliquots of unseparated digestion mixture (each, 1 μl, or approximately 1/10th of the total) were mixed at a 1:1 ratio with the matrix (saturated solution of 2,5-dihydroxybenzoic acid [Aldrich] in acetone). Samples were analyzed by collision-induced dissociation (CID) on a Micromass Autospec orthogonal acceleration TOF mass spectrometer (Micromass Inc.) equipped with an N_2 laser (337 nm). After the electric and magnetic sections (MS-1) were tuned manually to transmit the ^{12}C monoisotopic ion of the precursor mass, a two-stage deacceleration electrostatic lens focused the ions into an approximately parallel beam before they entered the gas collision cell (2). The collision cell was filled with Xe gas with a collision energy of 800 eV. Voltage applied periodically from a "push-out" electrode extracted the precursor and product ions into a linear TOF mass analyzer. All spectra were recorded with a microchannel plate detector by using a time-to-digital converter (Precision Instruments, Knoxville, Tenn.) (43).

Database searches for protein identification. A program available via the internet (http://prospector.ucsf.edu) and developed in the University of California—San Francisco (UCSF) Mass Spectrometry Facility (11b) was used to search genomic databases. The program, MS-Tag, uses fragment ion masses (generated by MALDI-PSD or -CID MS) to search the databases for matches to peptides from known proteins. The following parameters were used in the searches: no errors mode, *Mycobacterium* species, protein molecular mass range from 1,000 to 120,000 Da, trypsin digest (one missed cleavage allowed), parent ion mass tolerance of ± 1.5 Da, fragment ion mass tolerance of ± 1.5 Da, and allowed fragment ion types a, b, y, a-NH_3, b-NH_3, y-NH_3, b-H_2O, and internal. The protein sequences found by using MS-Tag were used to search protein databases for homologous proteins with NCBI's basic local alignment search tool (BLAST). Basic BLAST searches with the blastp program were performed on the nonredundant database (1). The Wisconsin sequence analysis package, version 8.0 (Genetics Computer Group, Inc., Madison, Wis.), was used to perform sequence alignments (PILEUP) and identity calculations (DISTANCES).

RESULTS AND DISCUSSION

Proteins of *M. tuberculosis* modulated by iron. Proteins of *M. tuberculosis* cultured in Sauton's medium containing a low concentration (1 μM) or a high concentration (70 μM) of iron were sequentially fractionated by ammonium sulfate precipitation (0 to 20%, 20 to 55%, and 55 to 95%) and analyzed by 2-D gel electrophoresis. With increasing amounts of ammonium sulfate, improved resolution of protein spots was obtained on 2-D gels. Heavy vertical and horizontal streaks were seen on 2-D gels with samples precipitated with 0 to 20% ammonium sulfate, and these streaks severely interfered with analysis of protein spots. (The streak lines are most likely caused by the presence in the sonicates of unusually large amounts of mycobacterial lipids that severely interfere with protein separation on 2-D gels.) Although Triton X-114 extraction improved the resolution of samples precipitated with 20 to 55% ammonium sulfate, the best comparison of protein

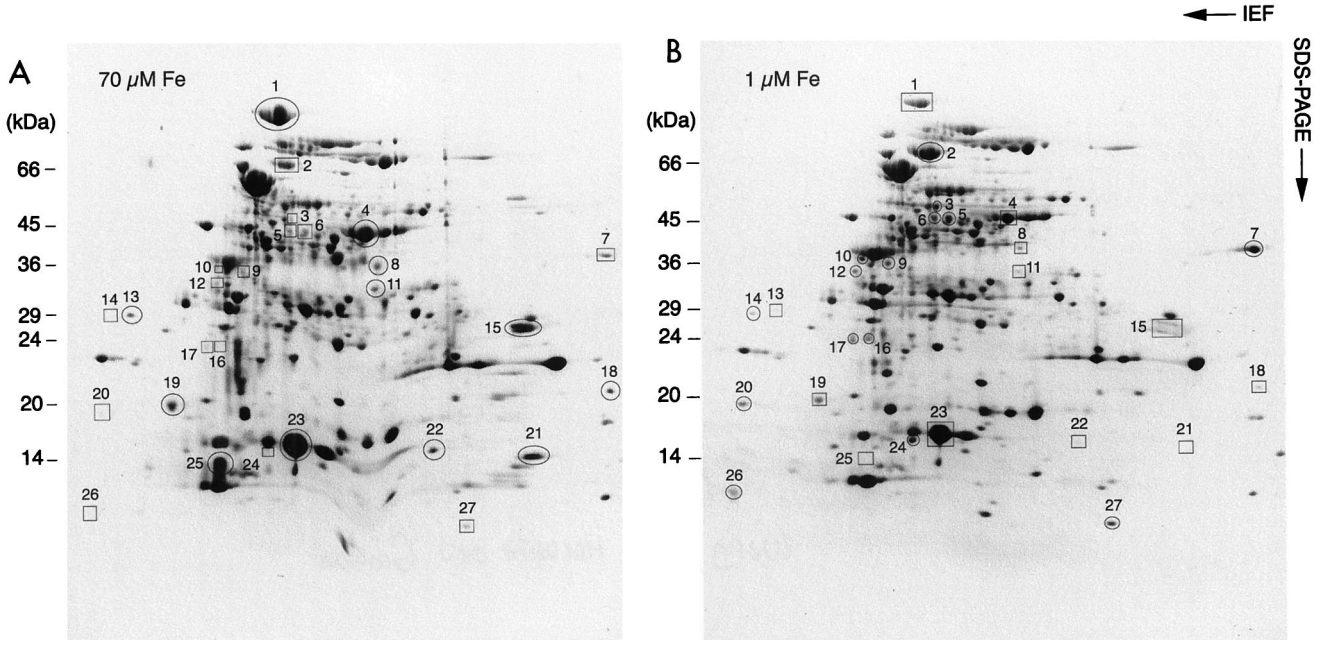

FIG. 1. 2-D gel analysis of proteins of *M. tuberculosis* cultured in medium containing a (A) high (70 μM) or (B) low (1 μM) concentration of iron. Equal amounts of proteins precipitated by 55 to 95% ammonium sulfate were separated by isoelectric focusing (pH 4 to 7 from left to right) in the first dimension and by linear gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 to 20% from top to bottom) in the second dimension. The second-dimension gels were stained with Coomassie blue. Open circles and open squares represent protein spots with increased or decreased expression under the indicated condition, respectively. The set of 2-D gels shown here is representative of the results of three independent experiments. The numbers are the protein gel spot numbers, as discussed in the text.

spots between high-iron-concentration and low-iron-concentration cultures was obtained from samples precipitated with 55 to 95% ammonium sulfate. Of more than 250 protein spots revealed by 2-D gel electrophoresis of samples precipitated with 55 to 95% ammonium sulfate, the expression of at least 15 proteins was induced and the expression of at least 12 proteins was decreased by low iron concentrations (Fig. 1). The protein spots with consistent differential expression from three different batches of low-iron-concentration and high-iron-concentration bacterial cultures were further analyzed by MALDI-MS.

Protein spots from single (spots 1, 2, 15, 18, 21, and 22 to 25) or several (spots 4, 11, and 21) 2-D gels were cut from the gels and digested with trypsin. The resulting digestion mixture was analyzed by MALDI-MS to determine the molecular masses of the tryptic peptides (Fig. 2A and Fig. 3A). To obtain sequence information, masses of the fragment ions of selected tryptic peptides were obtained by using MALDI-PSD (see Fig. 3B to D) or MALDI-CID (see Fig. 2B) MS. MS-Tag fragment ion searches with minimally restricted search parameters (*Mycobacterium* species and 1,000 to 120,000 Da) of the NCBI protein database offered possible matches to peptide sequences of *M. tuberculosis* proteins, although in some searches, the peptide fragmentation data also matched peptides in the same protein in an additional mycobacterium species (e.g., aconitase and elongation factor Tu [EF-Tu] [Table 1]). This information, in conjunction with the mass fingerprints of the proteins obtained by MALDI-TOF MS analysis, allowed for the matching of 11 protein spots to *M. tuberculosis* proteins in the database. BLAST searches of these protein sequences yielded possible identities and functional roles of many of these proteins, as summarized below and in Table 1.

Regulators. (i) Fur. Protein gel spot 25, whose expression was upregulated under high-iron-concentration conditions and virtually absent under low-iron-concentration conditions, was taken from a single 2-D gel. The gel plug was subjected to in-gel digestion with trypsin and the extracts were analyzed by MALDI-TOF MS to obtain molecular masses of the tryptic peptides. Analysis of the peptide with MH^+ at *m*/*z* of 1,410.8 by MALDI-CID MS and subsequent searching of the protein sequence databases with MS-Tag identified spot 25 as an *M. tuberculosis* Fur homolog. Fur (or ferric uptake regulator) proteins are a family of iron-responsive DNA-binding proteins. Subsequent review of the MALDI mass spectrum of the unseparated digestion mixture revealed two other tryptic peptides belonging to the *M. tuberculosis* Fur protein.

The level of iron in the environment is known to regulate the expression of genes coding for many high-affinity bacterial iron uptake pathways. Under iron-rich conditions, the Fur protein is activated when it binds Fe(II) as a cofactor. This activated repressor is then able to bind the "Fur box," a consensus sequence located in the promoter region of many bacterial genes. In conditions of iron deprivation, the Fur protein does not bind to the promoter sequence, allowing for the transcription of the genes (47). Homologs of the Fur repressor have been found in many gram-negative bacteria. Their sequences appear to be fairly well conserved, with a high degree of homology with the first-discovered *Escherichia coli* Fur protein, ranging from 49% homology for *Neisseria gonorrhoeae* Fur (4) to 76% for *Vibrio vulnificus* Fur (37). The Fur homolog of *M. tuberculosis* shows less identity to the Fur proteins of *E. coli* (22.9%), *Legionella pneumophila* (28.4%), and *N. gonorrhoeae* (25.4%). Originally thought of as only a negative repressor, Fur is now known to also positively regulate many genes in *E. coli* and *Salmonella typhimurium* (17, 59). Fur may also act as a global regulator affecting gene expression in response to signals besides iron levels. In addition, Fur may be part of a

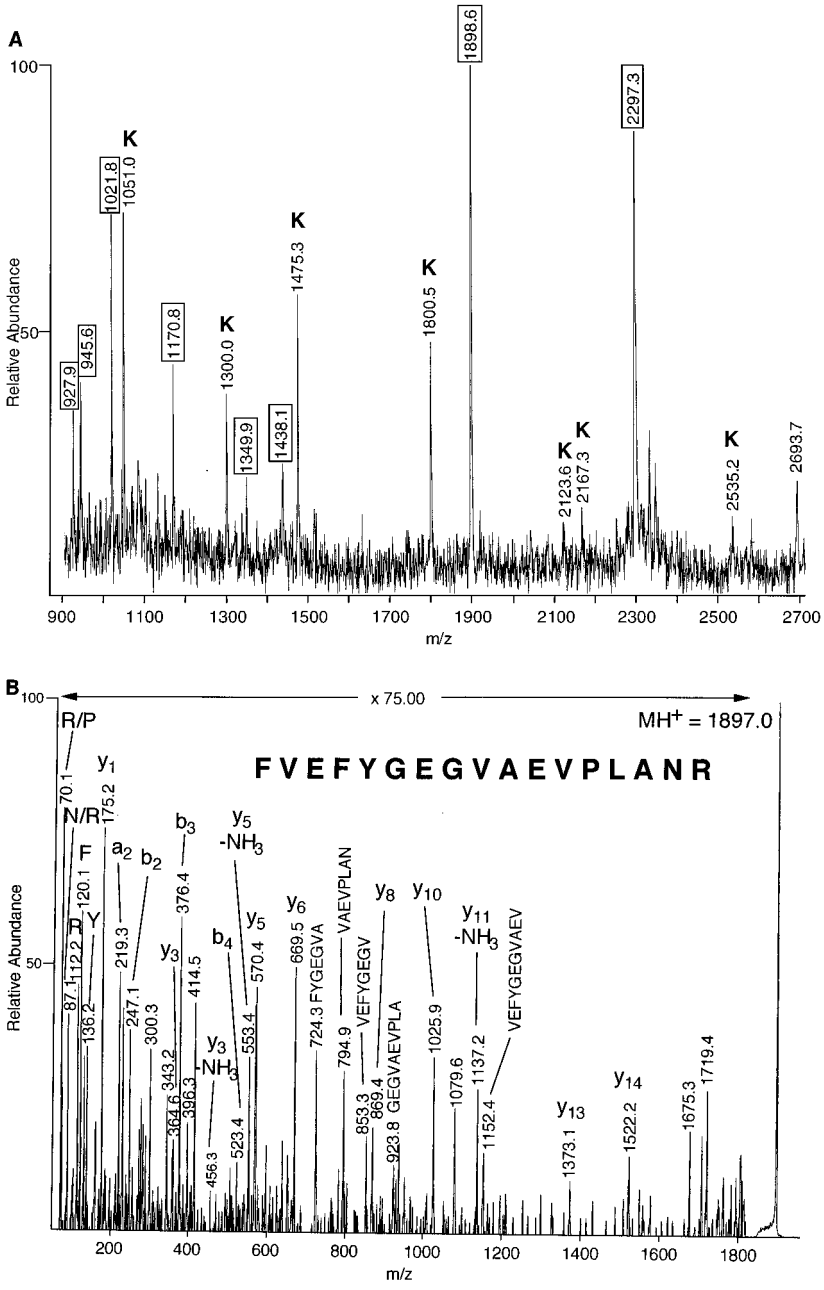

FIG. 2. Analysis of aconitase homolog by MS. (A) MALDI-TOF MS peptide mass fingerprint spectrum produced by in-gel tryptic digestion of gel spot 1. Masses outlined by boxes indicate tryptic peptides from the *M. tuberculosis* aconitase protein. Labeling of peaks with K indicates that they are tryptic peptides from contaminating human keratin. (B) MALDI-CID mass spectrum of a tryptic peptide with MH^+ at *m/z* of 1,897.0 (monoisotopic). Fragment ions referred to as a and b ions originate from peptide backbone cleavage with the charge retained on the N terminus, and y ions refer to peptide fragments with the charge retained on the C terminus (5). Multiple bond cleavages internal to the peptide are labeled with the corresponding portion of the peptide sequence. All (or a combination) of these fragment ions were used in the MS-Tag database searches.

cascade of control elements in which it regulates the expression of secondary regulatory elements (17).

(ii) Aconitase. Protein gel spot 1, which was expressed at a higher level under conditions of high iron concentration, was excised from a single 2-D gel and subjected to in-gel digestion with trypsin. MALDI-TOF MS analysis of the digestion extracts yielded molecular masses of several tryptic peptides (Fig. 2A). The peptide with MH^+ at *m/z* of 1,897.0 (monoisotopic) was analyzed by MALDI-CID MS to obtain additional sequence information (Fig. 2B). The resultant fragment ion data were used to search the protein databases by using MS-Tag, and the peptide was originally matched to a portion of a *Mycobacterium avium* protein (MW, 104,025.7 Da). A subsequent BLAST search of the nonredundant protein database with the *M. avium* peptide sequence revealed the protein to be an aconitase. The tight correlation of the fragmentation data with the *M. avium* aconitase peptide suggested that spot 1 was an *M. tuberculosis* aconitase protein whose sequence had yet to be entered into the database. Subsequent to our initial identification of this protein as an aconitase homolog, the sequence for

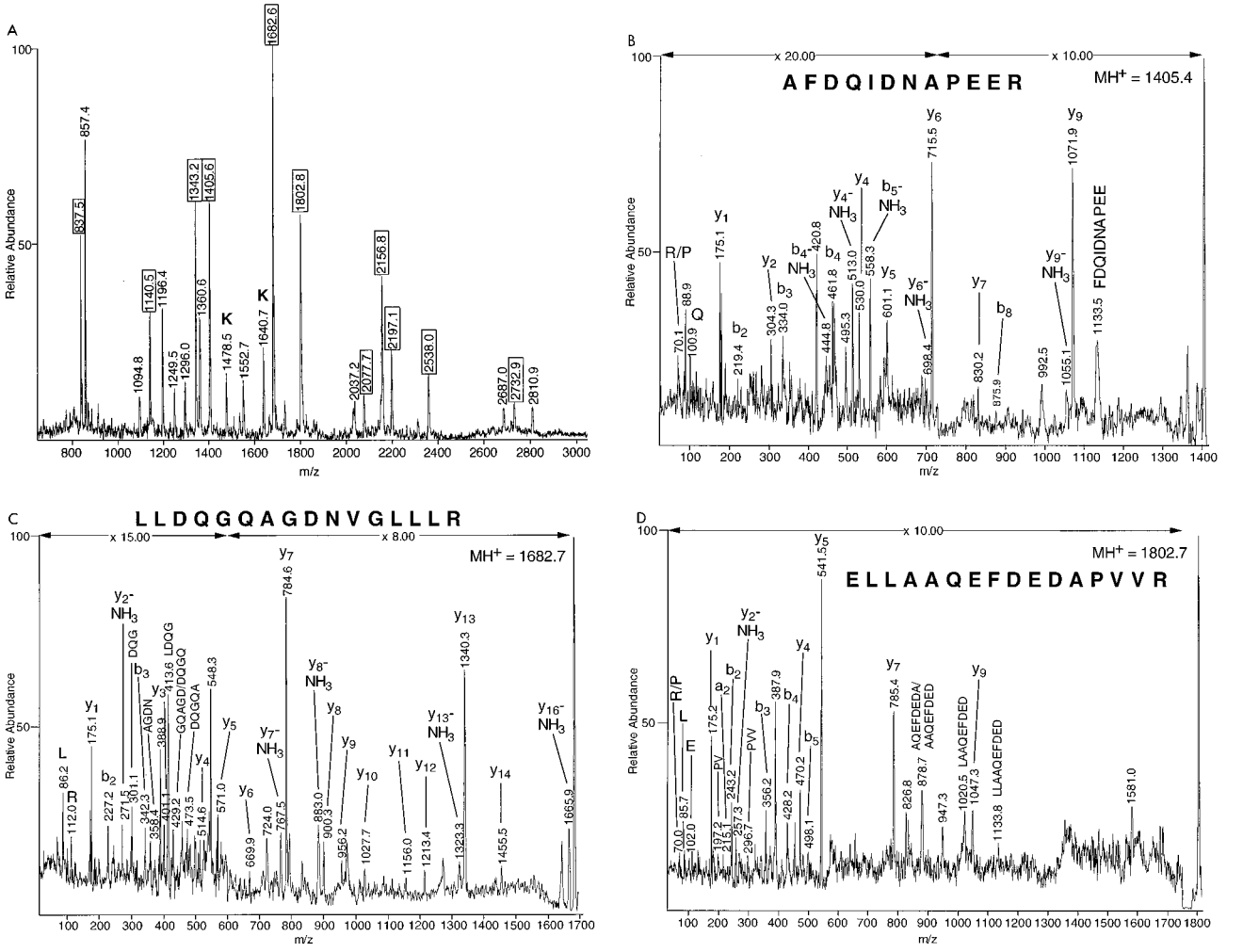

FIG. 3. Analysis of EF-Tu homolog (spot 4) by mass spectrometry. (A) MALDI-TOF MS peptide mass fingerprint spectrum produced by in-gel tryptic digestion of gel spot 4, pooled from four 2-D gels. (B through D) MALDI-PSD mass spectrum of a tryptic peptide with (B) MH^+ at *m/z* of 1,405.4, (C) MH^+ at *m/z* of 1,682.7, and (D) MH^+ at *m/z* of 1,802.7. Fragment ions detected in the PSD analysis were used to search the protein databases with MS-Tag. All three peptides were identified as belonging to EF-Tu proteins from both *M. tuberculosis* and *M. leprae*. In the mass fingerprint spectrum (panel A) the peak at *m/z* of 857.4 was identified as SQRYFR from MALDI-PSD data and was identified as the sole peptide in spot 4 belonging to a minor contaminating protein (NCBI accession no. 1722931) of unknown function. See legend to Fig. 2 for an explanation of peak labels.

the *M. tuberculosis* aconitase protein (MW, 102,449.6 Da) was entered; it contains a peptide (FVEFYGEGVAEVPLANR) whose sequence is identical to that of the *M. avium* peptide that was originally discovered by MS-Tag. A total of eight tryptic peptides from the *M. tuberculosis* aconitase were detected in the MALDI mass spectrum of the unseparated digest. The *M. tuberculosis* aconitase protein is highly homologous to the *M. avium* aconitase (83.1% identity) and to a lesser extent to the aconitases from *E. coli* (58.3% identity) and the mouse (51.0% identity).

It is not surprising that an aconitase was identified as one of the iron-regulated proteins in these studies. The cytosolic aconitase is a protein with dual roles. It catalyzes the reversible isomerization of citrate and isocitrate via *cis*-aconitate, as part of the Krebs cycle, and serves as an iron-responsive element (IRE) binding protein. Aconitases are monomeric proteins that contain a single cubane (4Fe-4S) cluster. In the large family of 4Fe-4S proteins, it is unusual, in that only three of the irons are directly ligated to the peptide backbone through cysteines. In aconitase, the fourth iron is labile and coordinates the atoms of the cluster along with a water molecule and the substrate. The instability of the cluster is exploited as a molecular switch, enabling cells to reciprocally regulate the aconitase and RNA binding activity of the protein in response to changes in iron levels. Under conditions of iron deprivation, the apo form (with no 4Fe-4S cluster) of the protein is inactive as an aconitase but active for RNA binding. The IRE-binding function of the protein results in a tight interaction with the IREs contained in the mRNAs of molecules involved in iron storage in mammalian cells, like transferrin receptor and ferritin. Binding of the protein to IREs located in the 5′ untranslated region of mRNAs prevents translation by inhibiting the binding of initiation factors. However, binding of the protein to IREs in the 3′ untranslated region stimulates mRNA translation by protecting the mRNA against degradation (45). When iron levels rise, the protein dissociates from the IRE and is again active as an aconitase. The mechanism underlying the physiological role of the cytosolic aconitase in iron regulation is still unclear. The fact that citrate, the substrate for the aconitase, is capable of binding iron and has been proposed as a possible

TABLE 1. Summary of proteins identified from 2-D gels of *M. tuberculosis* cell extracts

Spot no.	Name (NCBI accession no.), MW	Peptide MH^+ (Da)	Δ in mass	Start-end position	Sequence[b]
1	Aconitase (NCBI 2791409), 102,449.6 Da	927.9	0.3	42–49	VLAENLLR
		845.6	0.2	747–754	DYNSFGSR
		1,021.8	0.3	839–847	AVIAESFER
		1,349.9	0.2	904–916	GDGATIEFDAVVR
		1,438.1	0.4	773–785	NQLLDDVSGGYTR
		1,897.0	0.5	278–294	*FVEFYGEGVAEVPLANR*[e]
		2,297.3[a]	0.8	667–686	NLPTPSGNTFEWDPNSTYVR
2	PEPCK (NCBI 1871584), 67,253.5 Da	688.3	0	524–528	WIVDR
		957.6	0.2	371–377	**GNDWYFR**
		1,086.7	0.1	423–432	TTVPLVTEAR
		1,114.7	0.1	496–503	VFFVNWFR
		1,163.7	0.1	203–211	**YITHFPETR**
		1,310.4	0.8	510–520	FLWPGFGENSR
		1,377.8	0.2	290–301	AETLGDDIAWMR
		1,544.1	0.4	165–178	AALEKMGDDGFFVK[c]
		1,544.1	0.3	110–121	SIMKDLYRGCMR
4	EF-Tu (NCBI 399422), 43,593.8	837.5	0	120–126	EHVLLAR
		1,140.5	0.07	256–265	TTVTGVEMFR
		1,343.2	1.5	365–376	LIQPVAMDEGLR
		1,405.6	1	48–59	**AFDQIDNAPEER**[f]
		1,682.6	0.7	267–282	**LLDQGQAGDNVGLLLR**[f]
		1,802.8[a]	0.2	158–173	**ELLAAQEFDEDAPVVR**[f]
		2,077.2[a]	0.9	140–157	ADAVDDEELLELVEMEVR
		2,156.8[a]	1.3	236–255	GVINVNEEVEIVGIRPSTTK
		2,197.1[a]	0.6	207–225	ETDKPFLMPVEDVFTITGR
		2,538.0[a]	1.9	233–255	VERGVINVNEEVEIVGIRPSTTR
		2,732.9[a]	1.7	93–119	NMITGAAQMDGAILVVAATDGPMPQTR
11	Oxidoreductase (NCBI 2072661), 29,814.6	970.3	0.8	7–15	TMFISGASR
		1,193.5	0.9	113–122	**FDLMNGIQVR**
		1,209.5	0.9	113–122	FDLMNGIQVR[c]
		1,768.7[a]	0.7	55–71	ELEEAGGQALPIVGDOR
		2,037.7[a]	1.4	194–213	TMVATAAVQNLLGGDEAMAR[c]
		2,530.6[a]	1.7	216–238	KPEVYADAAYVIVNKPATEYTGK
15	Hypothetical 21.5-kDa protein (NCBI 1731190), 21,534.6	866.3	0.3	75–81	FTAEELR
		922.3	0.1	95–101	YNELVER
		1,239.5	0.9	83–94	AAEGYLEAATSR
		1,584.6[a]	0.2	60–72	**LQEDLPEQLTELR**
		1,648.2[a]	0.6	95–108	**YNELVERGEAALER**
		2,538.7[a]	0.9	122–145	AEGYVDQAVELTQEALGTVASQTR
18	PPIase (NCBI 1552563), 19,239.5	1,602.7	0	141–154	**HTIFGEVIDAESQR**
		2,203.8[a]	1.4	162–182	**TATDGNDRPTDPVVIESITIS**
		2,431.2[a]	0.3	51–73	DYSTQNASGGPSGPFYDGAVFHR
21	Lsr2 (NCBI 2113979), 12,098.5	770.5	0.1	50–56	QWVAAGR[d]
		787.5	0.1	50–56	**QWVAAGR**
		884.6	0.2	90–97	NGHNVSTR
		1,040.7	0.2	89–97	RNGHNVSTR
		1,286.8	0.1	73–84	GAIDREQSAAIR
		1,570.4	0.6	98–112	**GRIPADVIDAYHAAT**
22	Hypothetical 15.9-kDa protein (NCBI 2113920), 15,312.4	1,113.0	0.4	124–134	**LLGSVPANVSR**
		1,629.4[a]	0.5	60–74	VTGTAPIYEILHDAK
		1,656.1[a]	0.2	107–123	ADLLVVGNVGLSTIAGR
		1,698.0[a]	0.1	34–48	**LIIASAYLPQHEDAR**
		2,478.7[a]	0.1	84–106	NVEERPIVGAPVDALVNLADEEK
23, 24	HSP 16.3 (α-crystallin) (NCBI 231343), 16,227.4	1,163.0	0.5	91–100	**SEFAYGSFVR**
25	Fur (NCBI 231343), 15,892.0	1,410.9	1.2	1–12	*MSSIPDYAEQLR*
		1,450.1	0.4	72–84	IQPSGSVARYESR
		1,577.1	0.7	71–84	KIQPSGSVARYESR

[a] MW values indicate average masses (all other masses are monoisotopic).
[b] Sequences in boldface type were determined by MALDI-PSD. Sequences in italic type were determined by MALDI-CID.
[c] Contains one oxidized methionine.
[d] Contains N-terminal pyro-glutamic acid.
[e] MS-Tag search by using this sequence found *M. tuberculosis* and *M. avium* aconitase proteins containing this peptide.
[f] MS-Tag searches by using these sequences found *M. tuberculosis* and *M. leprae* EF-Tu proteins containing these peptides.

transporter of intracellular iron in mammals is very curious. In one model, the increased synthesis of iron storage proteins (ferritin and transferrin) and reduced synthesis of iron uptake proteins (transferrin receptors) in iron-replete conditions, in addition to the reduced levels of citrate (conversion to isocitrate by aconitase), eventually lead to reduced intracellular iron levels and the subsequent conversion of the protein back to its iron-binding form (44).

(iii) EF-Tu. Protein gel spot 4, which is expressed at higher levels when grown in the high-iron-concentration medium, was excised from four 2-D gels and subjected to in-gel tryptic digestion. The resultant digestion mixture was analyzed by MALDI-TOF MS (Fig. 3A). The tryptic peptides with MH^+ at *m*/*z* of 1,405.6 (Fig. 3B), 1,682.6 (Fig. 3C), and 1,802.8 (Fig. 3D) were further analyzed by MALDI-PSD MS to obtain sequence information. Database searching by using MS-Tag and BLAST revealed spot 4 to be EF-Tu, a helper protein involved in protein synthesis encoded by the *tuf* gene. A total of 11 tryptic peptides from *M. tuberculosis* EF-Tu were observed, and additional sequence information was obtained on three of the peptides (see Fig. 3). EF-Tu is a GTPase which promotes the binding of aminoacyl-tRNA to ribosomes (58). The *tuf* gene of *M. tuberculosis* was discovered when a λgt11 *M. tuberculosis* gene library was screened with monoclonal antibodies raised by immunizing rats with live *Mycobacterium bovis* bacillus Calmette-Guerín. The *M. tuberculosis* EF-Tu homolog showed high sequence similarity with EF-Tu proteins from several other organisms, including *Mycobacterium leprae* (95.2% identity), *S. typhimurium* (75.1% identity), and *E. coli* (75.1% identity).

Besides its essential role in protein biosynthesis, EF-Tu has been shown by Young and Bernlohr to be methylated and to become membrane associated when *E. coli* is starved for glucose, galactose, ammonia, glutamate, or phosphate (63). This raises the possibility that EF-Tu may also have roles in the regulation of cell growth and the organism's response to stress. These investigators propose that EF-Tu's membrane association allows for its interaction with receptors or proteins that interact with nutrients in the environment that could regulate its methylation. Conditions of nutrient deprivation would result in EF-Tu hypermethylation and subsequent membrane release, allowing for the possibility that the intracellular EF-Tu assumes multiple regulatory roles. In addition to regulating translation through its interaction with tRNA and ribosomes, enabling it to stop the translation of unnecessary proteins and trigger the synthesis of stress-induced proteins, EF-Tu has also been known to act as a transcriptional activator in the presence of RNA polymerase and the appropriate sigma factor (21, 60). Therefore, it may be able to regulate both the translation and transcription of starvation-induced proteins. A preliminary report that patients with tuberculosis but not tuberculin-negative individuals develop an antibody response to the protein suggests a potential role for the protein in serodiagnosis of mycobacterial disease (9).

Antigens. (i) LSR2. Protein gel spot 21, expressed at a higher level under high-iron-concentration conditions, was excised from four 2-D gels. In-gel tryptic digestion, followed by MALDI-MS analysis, revealed gel spot 21 to be an *M. tuberculosis* homolog of LSR2, a protein antigen of *M. leprae*. The digestion mixture was found to contain six tryptic peptides. Additional sequence information was obtained by using MALDI-PSD MS on two of the peptides.

Using polyclonal antibodies from pooled sera of lepromatous patients, Laal et al. (31) screened a λgt11 DNA expression library in an effort to identify genes involved in the immune response to *M. leprae* infection. These investigators identified LSR2, a dominant T-cell antigen. BLAST searches of this ~10-kDa protein revealed that *M. tuberculosis* LSR2 has 92.9% identity with the LSR protein of *M. leprae* but is not homologous to any other known proteins. Analysis of overlapping peptides spanning the *M. leprae* LSR sequence showed that two peptides (GVTYEIDLTNKNAA and IDLTNKNA AKLRGD) were recognized by the sera of leprosy patients (56). Single-residue deletions of the peptides enabled the identification of three distinct sequences (GVTY, NAA, and RGD) found to be important for antibody recognition (55). Although nothing is yet known of the *M. tuberculosis* homolog's role in the immune response, two of the three sequences important for antibody recognition in leprosy patients, GVTY and RGD, are present in its sequence.

(ii) Hsp16.3 (α-crystallin homolog). Protein gel spot 23, upregulated under conditions of high iron concentration, was taken from a single 2-D gel and subjected to in-gel digestion and MALDI-MS analysis. Only one *M. tuberculosis* tryptic peptide was identified in the digestion mixture. MALDI-PSD MS analysis on this peptide (MH^+ at *m*/*z* of 1,163.0) and database searching revealed gel spot 23 to be a small heat shock protein (Hsp16.3) of the α-crystallin family (61). Mass spectrometric analysis showed gel spot 24 also to have tryptic peptides originating from Hsp16.3, possibly a degraded and/or truncated form of the protein. Members of this family of small heat shock proteins are thought to function as chaperones, acting as molecular surfactants which prevent protein aggregation through nonspecific weak interactions with the properly folded proteins (10, 36). Hsp16.3 is a major *M. tuberculosis* antigen which can generate a cell-mediated immune response and is thought to be located on the periphery of the cell membrane (27, 32). When Hsp16.3 was overexpressed in wild-type *M. tuberculosis*, a slower decline in viability after the end of log-phase growth was observed (64). Besides the *M. tuberculosis* Hsp16.3, an α-crystallin homolog has been detected in *M. leprae* (46) and *M. bovis* but not in *Mycobacterium smegmatis* or the pathogenic species *M. avium* (64).

In investigations into the regulation of Hsp16.3 expression under various stress conditions, carbon starvation, heat and cold shock, and low pH all failed to induce Hsp16.3 expression (64). The only environmental stress shown to significantly upregulate the expression of Hsp16.3 was growth under microaerobic or anaerobic conditions (12, 64). The environment inside caseous granulomas, where mycobacteria are thought to exist in a dormant state, is unknown. However, many environmental stresses, like oxygen deprivation, may signal mycobacteria to produce proteins like the α-crystallin homolog to aid in the long-term survival of the bacteria. On the other hand, the diversity of this family of small heat shock proteins suggests that the protective capacity of this protein may be general and not necessarily specific to the pathogenic species *M. tuberculosis* and *M. leprae*.

Enzymes. (i) PEPCK. Protein gel spot 2, which was upregulated under low-iron-concentration conditions, was found to be homologous to many GTP-dependent phosphoenolpyruvate (PEP) carboxykinases (PEPCK) from numerous other species, including *M. leprae* (86.0% identity), *Drosophila melanogaster* (51.3% identity), and *Homo sapiens* (52.5% identity). MALDI-MS analysis of the digestion mixture revealed nine tryptic peptides. Further analysis with MALDI-PSD MS was carried out on two of the peptides to obtain sequence information. PEPCK is part of the gluconeogenic pathway, catalyzing the reversible decarboxylation and mononucleotide-dependent phosphorylation of oxaloacetate PEP (42). Most PEPCKs require two metal cations for activity. One of the cations (Mg^{2+} or Mn^{2+}) must complex with the substrate to

form a cation-nucleotide complex. For optimal activity, a second cation (often a transition metal) is required to interact directly with the protein, possibly mediating the interaction of the substrate (oxaloacetate or PEP) with the enzyme to facilitate the formation of the active ternary complex (34). In GTP-dependent PEPCKs, it has been proposed that the second ion may help position the substrate for catalysis by binding the PEP phosphoryl group and the nucleotide β- or γ-phosphate, either directly (23) or through an interaction with water (13, 35, 42).

In early investigations, Fe(II) was found to be the most efficient activator of rat liver cytosolic PEPCK when incubated with the cytosol fraction of the liver homogenate (3). However, studies found a rapid Fe(II)-dependent inactivation after continued incubation with the enzyme in the absence of substrate. This loss of activity is thought to result from PEPCK oxidative damage caused by the reactive oxygen species formed from Fe(II) autoxidation (52). The presence of a ferroactivator, a cytosolic protein factor originally identified as glutathione peroxidase in rat liver and thought to act by removing reactive oxygen species, results in the restoration of enzyme activity (50). It has been proposed that PEPCK may exist in a dynamic equilibrium between an active and inactive form (caused by the Fe(II) autoxidation). If this is the case, the ferroactivator may have a role in shifting the equilibrium towards the active enzyme (52).

(ii) Oxidoreductase. Protein gel spot 11, which was downregulated in low-iron-concentration medium, was identified as a homolog of an oxidoreductase. A total of six tryptic peptides was detected by MALDI-MS analysis of the digestion mixture. The large number of microbial alcohol oxidoreductases can be categorized into three major groups: (i) NADP-dependent dehydrogenases, (ii) NADP-independent enzymes which use pyrroloquinoline quinone, heme, or cofactor F_{420} as the cofactor, and (iii) enzymes that catalyze the essentially irreversible oxidation of alcohols (51). The *M. tuberculosis* oxidoreductase homolog belongs to a subgroup of the first group, NADP-dependent dehydrogenases, the short-chain alcohol dehydrogenase superfamily. Members of this subgroup are known to act on a large variety of substrates, including sugars, steroids, prostaglandins, aromatic hydrocarbons, antibiotics, and compounds involved in nitrogen metabolism (30). The short-chain alcohol dehydrogenase enzymes do not require any metal ions to function and are typically around 250 amino acids in length. Sequence comparisons between members of the superfamily reveal six conserved domains (30). Among the family members, there is a pattern that 13 residues are largely conserved. Alignments between enzyme pairs typically reveal approximately 25% identity, with the identities for single forms ranging from 14 to 58% (48). The oxidoreductase of *M. tuberculosis* shows homology to enzymes from *Caenorhabditis elegans* (48% identity), *E. coli* (27.6% identity), and *H. sapiens* (19.5% identity) within this range.

(iii) PPIase. Gel spot 18, whose expression is reduced in a low-iron-concentration environment, was removed from a single 2-D gel. MALDI-PSD MS analysis of two of the three tryptic peptides found in the digestion mixture (MH^+ at *m/z* of 1,602.7 and 2,203.8) followed by database searching matched them to an *M. tuberculosis* protein (MW, 19,239.5) with significant homology to peptidyl-prolyl *cis-trans* isomerases (PPIase).

Immunophilins are housekeeping proteins with many roles, including membrane channeling and protein folding and trafficking (15). Besides exhibiting PPIase activity in the unliganded form, immunophilin-drug (cyclophilin or FK506) complexes inhibit clonal expansion of T cells and have toxic effects on numerous other cellular components. Many intracellular pathogens produce proteins with significant homology to immunophilins and have PPIase activity. The role of these proteins in microbial pathogenicity is as yet unclear; the immunophilins may interact with various partner molecules in mammalian cells or may interact with other components through their PPIase activities by interrupting protein folding or altering protein structure dynamics (20). Many facultative or obligate intracellular pathogens, for example *L. pneumophila* and *Chlamydia trachomatis*, produce FK506-binding protein (FKBP)-like immunophilins, such as the Mip (macrophage infectivity potentiator) protein of *Legionella* spp. These proteins have been proposed to aid in intracellular survival. Mip-protein-negative mutants appear to have a reduced ability to initiate intracellular replication (11). The site of action of Mip protein is not known, nor is it known whether it acts by altering the conformation of other *Legionella* proteins (20).

The *M. tuberculosis* PPIase shows homology to immunophilins of the cyclophilin family of several other species including *Streptomyces chrysomallus* (61.4% identity), *C. elegans* (48.4% identity), and *H. sapiens* (46.1% identity). Unlike the Mip proteins of the FKBP family, these bacterial proteins have not been extensively characterized. However, the existence of cyclophilin-like proteins in *E. coli*, *S. typhimurium*, and *L. pneumophila* suggests that a large array of these proteins is produced by bacterial species. There is speculation that these proteins have roles in protein folding and secretion (38).

CONCLUSION

In this study, we have used 2-D gel electrophoresis, MALDI-MS, and database searching to identify *M. tuberculosis* proteins regulated by extracellular iron levels. Previous studies in our laboratories with 1-D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (unpublished data) did not allow for the direct identification of iron-regulated proteins, since single bands were found to contain multiple comigrating proteins. However, once we obtained the proper conditions for the separation of complex protein mixtures from *M. tuberculosis* cell lysates by 2-D gel electrophoresis, we were able to analyze and identify single-protein-containing gel spots by MS. The number of *M. tuberculosis* tryptic peptides detected in digestion mixtures from individual samples varied greatly, ranging from as few as 1 to as many as 11 peptides (Table 1). Therefore, in addition to determining the masses of the tryptic peptides, we chose to generate sequence information. In most cases, useful sequence data could be obtained from only the more abundant signals in the MALDI mass spectra of the digestion mixtures by PSD and/or CID analysis. This sequence data provided a higher level of confidence in the identification of proteins than would have been afforded by tryptic peptide masses alone. This was particularly true in cases where only a few peptide molecular masses could readily be observed over background.

2-D gel electrophoresis identified at least 27 proteins whose expression is modulated by iron concentration—15 proteins are upregulated and 12 proteins are downregulated under low-iron-concentration conditions. Of these proteins, 11 (including two forms of 1 protein) were identified by MALDI-MS and database searching. Two of these proteins (gel spots 15 and 22) were not found to be homologous to any proteins of known function and are listed as hypothetical proteins in Table 1. The identification of two of the proteins as Fur and aconitase homologs suggests the possibility that these proteins function as transcriptional regulators in *M. tuberculosis*, as in other bacteria, and that they exert control over the expression of many of the other proteins which show differential expression under low- and high-iron-concentration conditions. In future experi-

ments, this may be explored further by comparing the protein expression of Fur or aconitase deletion mutants with wild-type *M. tuberculosis* or by analyzing genes with upstream Fur or aconitase binding regions. EF-Tu may also play a role in the organism's response to iron starvation conditions through its ability to regulate protein expression.

Despite the successful identification of 10 proteins in this study, we were unable to obtain sufficient data to identify unambiguously 16 of the proteins that appeared to be under iron regulation. Further refinements in methodology should allow us to identify these latter proteins in addition to other proteins whose expression levels are very low and may be visualized only by silver staining. Alternatively, the application of DNA microarray technologies (for a review, see reference 40) could provide information at the level of RNA message. While such information does not necessarily correlate with changes in protein expression, it may be useful for comparative purposes and/or to suggest additional protein candidates for further investigation by the 2-D gel electrophoresis approach.

Our study demonstrates the feasibility and utility of combining three powerful technologies—2-D gel electrophoresis, MS, and database searching—to study how mycobacteria and other pathogens respond to changes in the environment. Studies of this type should help us to take full advantage of the wealth of new data provided by genomic studies and greatly enhance our understanding of the pathophysiology of *M. tuberculosis* and other human pathogens.

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ADDENDUM IN PROOF

Since the submission of this manuscript, the sequence of the complete genome of *Mycobacterium tuberculosis* has been published (S. T. Cole et al., Nature **393:**537–544, 1998.)

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