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Definition of *Mycobacterium tuberculosis* Culture Filtrate Proteins by Two-Dimensional Polyacrylamide Gel Electrophoresis, N-Terminal Amino Acid Sequencing, and Electrospray Mass Spectrometry

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A number of the culture filtrate proteins secreted by *Mycobacterium tuberculosis* are known to contribute to the immunology of tuberculosis and to possess enzymatic activities associated with pathogenicity. However, a complete analysis of the protein composition of this fraction has been lacking. By using two-dimensional polyacrylamide gel electrophoresis, detailed maps of the culture filtrate proteins of *M. tuberculosis* H37Rv were generated. In total, 205 protein spots were observed. The coupling of this electrophoretic technique with Western blot analysis allowed the identification and mapping of 32 proteins. Further molecular characterization of abundant proteins within this fraction was achieved by N-terminal amino acid sequencing and liquid chromatography-mass spectrometry. Eighteen proteins were subjected to N-group analysis; of these, only 10 could be sequenced by Edman degradation. Among the most interesting were a novel 52-kDa protein demonstrating significant homology to an α-hydroxysteroid dehydrogenase of *Eubacterium* sp. strain VPI 12708, a 25-kDa protein corresponding to open reading frame 28 of the *M. tuberculosis* cosmid MTCY1A11, and a 31-kDa protein exhibiting an amino acid sequence identical to that of antigen 85A and 85B. This latter product migrated with an isoelectric point between those of antigen 85A and 85C but did not react with the antibody specific for this complex, suggesting that there is a fourth member of the antigen 85 complex. Novel N-terminal amino acid sequences were obtained for three additional culture filtrate proteins; however, these did not yield significant homology to known protein sequences. A protein cluster of 85 to 88 kDa, recognized by the monoclonal antibodies IT-57 and IT-42 and known to react with sera from a large proportion of tuberculosis patients, was refractory to N-group analysis. Nevertheless, mass spectrometry of peptides obtained from one member of this complex identified it as the *M. tuberculosis* KatG catalase/peroxidase. Thus, the detailed mapping of *M. tuberculosis* proteins, combined with state-of-the-art analytical techniques such as mass spectrometry, provides a basis for further analysis and rapid identification of biologically relevant molecules.

In vitro cultivation of *Mycobacterium tuberculosis* results in the accumulation in the extracellular milieu of a complex set of proteins collectively termed the culture filtrate proteins (CFPs). The most notable feature of this protein fraction is its immunodominance. Seibert and Munday (51) were the first to provide evidence that the culture filtrate is a major repository of antigens involved in the protective immune response and to provide biochemical definition of this fraction. More recently, as reviewed by Andersen (1), several investigators have argued that the dichotomous immune responses engendered by vaccination of experimental animals with live versus heat-killed bacilli are attributable to the active secretion of CFPs by viable *M. tuberculosis*. This hypothesis is supported by more recent studies demonstrating the ability of *M. tuberculosis* CFPs to induce a protective T-cell response (4, 25, 43, 46). Attempts to define the immunologically active components within this fraction have led to the purification and characterization of several proteins including the 6-kDa ESAT6 (56), 24-kDa MPT64 (39, 47), 30- to 31-kDa Ag85 complex (21, 24, 39), and 45-kDa MPT32 (15, 39, 48). A strong humoral response against CFPs has also been noted (29), and antigens such as the 45-kDa MPT32 (18), the 38-kDa PstS homolog (9), and an 88-kDa protein complex (29, 49) hold promise as tools for serodiagnosis.

To date, the most extensive characterization of the CFPs of *M. tuberculosis* is that by Nagai et al. (39), in which 12 major proteins were purified, partially characterized, and mapped by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). In addition, several other proteins, primarily those defined by monoclonal antibody (MAb) reactivity, have been located within culture filtrate preparations (17, 26). Along with actively secreted proteins, the culture filtrate possesses somatic molecules that are released into the medium during replication or by autolysis (5, 39, 63). As demonstrated by Andersen et al. (5), the protein profile of the culture filtrate is highly dependent on the cultivation time. Furthermore, the medium used and factors such as temperature and shaking may also affect the profile of proteins in the culture filtrate (5, 34). Thus, due to variations in the protocols used for preparation of CFPs, an absolute understanding of the protein composition of this fraction is difficult to obtain from the current literature.

Thus, in this report, we combined 2-D PAGE, Western blot analysis, N-terminal amino acid sequencing, and liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) to develop a detailed map of CFPs and obtained partial amino acid sequences for six previously undefined, relatively abundant proteins within this fraction. In all, this work provides a detailed portrait of the protein profile of this immunologically important fraction and a spectrum of proteins to which those from clinical isolates of *M. tuberculosis* can be compared. The applicability of this approach to defining the major antigens recognized by circulating antibodies in tuberculosis patients is presented separately (49).

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MATERIALS AND METHODS

Growth of *M. tuberculosis* and preparation of culture filtrate proteins. *M. tuberculosis* H37Rv (ATCC 27294) was obtained from the American Type Culture Collection (Rockville, Md.). Initially, this strain was inoculated from a 1-ml frozen stock of approximately 10^8 CFU/ml into 10 ml of glycerol-alanine-salts (GAS) medium (58); three such cultures were prepared. After incubation at 37°C for 14 days with gentle agitation, each 10-ml culture was passed two more times, increasing the volume of medium 10 times for each pass. The resulting 1-liter cultures were termed pass no. 4, and 3 liters of pass no. 4 cultures were used to inoculate 30 liters of GAS medium. After 14 days of growth at 37°C with gentle agitation, the culture supernatant was removed from the cells by filtration and the CFPs were concentrated and processed as described previously (14). The protein content of the concentrated culture filtrate was quantitated by the bicinchoninic acid protein assay (55).

To establish a growth curve for *M. tuberculosis* H37Rv, culture tubes (13 by 100 mm) containing 3 ml of GAS medium with 0.05% Tween 80 were inoculated with actively growing *M. tuberculosis* cultures to an optical density of 0.1 at 600 nm. These cultures were incubated at 37°C with stirring, and the optical densities at 600 nm were measured approximately every 24 h for a 22-day period.

Antibodies. MAbs IT-69 (HBT11) and IT-67 (L24.b4) were obtained from Ase B. Andersen, Statens Seruminstitut, Copenhagen, Denmark. MAb A3h4 was obtained from P. K. Das and A. Rambukana, University of Amsterdam, Amsterdam, The Netherlands, and MAbs F126-2 and HYB 76-8 were obtained from A. H. J. Kolk, Royal Tropical Institute, Amsterdam, The Netherlands, and I. Rosenkrands, Statens Seruminstitut, Copenhagen, Denmark, respectively. All other MAbs were supplied through the WHO Monoclonal Antibody Bank maintained by T. M. Shinnick, Centers for Disease Control, Atlanta, Ga. Anti-MPT63 polyclonal serum was provided by H. G. Wiker and M. Harboe, University of Oslo, Oslo, Norway. S. Nagai provided polyclonal sera specific for MPT 32, MPT 35, MPT 46, and MPT 57. A list of these antibodies and their reactivity to the CFPs of *M. tuberculosis* H37Rv is presented in Table 1.

SDS-PAGE and 2-D PAGE of culture filtrate protein. Sodium dodecyl sulfate (SDS)-PAGE was performed under reducing conditions by the method of Laemmli (30) with gels (7.5 cm by 10 cm by 0.75 mm) containing a 6% stack over a 15% resolving gel. Each gel was run at 10 mA for 15 min followed by 15 mA for 1.5 h.

Proteins were separated by 2-D PAGE by the method of O'Farrell (41) with minor modifications. Specifically, 70 μg of CFP was dried, suspended in 30 μl of isoelectric focusing sample buffer (9 M urea, 2% Nonidet P-40, 5% β-mercaptoethanol, 5% Pharmalytes [pH 3 to 10; Pharmacia Biotech, Piscataway, N.J.]), and incubated for 3 h at 20°C. An aliquot of 25 μg of protein was applied to a 6% polyacrylamide isoelectric focusing tube gel (1.5 mm by 6.5 cm) containing 5% Pharmalytes (pH 3 to 10 and 4 to 6.5 in a ratio of 1:4). The proteins were focused for 3 h at 1 kV with 10 mM H_3PO_4 and 20 mM NaOH as the catholyte and anolyte, respectively. The tube gels were subsequently immersed in sample transfer buffer (16) for 30 min and placed on a preparative SDS-polyacrylamide gel (7.5 cm by 10 cm by 1.5 mm) containing a 6% stack over a 15% resolving gel. Electrophoresis in the second dimension was carried out at 20 mA per gel for 0.3 h followed by 30 mA per gel for 1.8 h. Proteins were visualized by staining with silver nitrate (38).

Computer-aided analysis of 2-D gels. Silver stained 2-D PAGE gels were imaged with a cooled charge-coupled device digitizing camera and analyzed with MicroScan 1000 2-D gel analysis software for Windows 3.0 (Technology Resources, Inc., Nashville, Tenn.). Protein peak localization and analysis were conducted with a minimum allowable peak height of 1.0 and a minimum allowable peak area of 2.0.

Western blot analyses. Proteins, subjected to 2-D PAGE or SDS-PAGE, were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) (59) which were blocked with 1.0% bovine serum albumin in 0.05 M Tris-HCl (pH 7.5)–0.15 M NaCl–0.05% Tween 80 (TBST). These membranes were incubated for 2 h with specific antibodies diluted with TBST to the proper working concentrations (Table 1). After being washed, the membranes were incubated for 1 h with anti-mouse or anti-rabbit alkaline phosphatase-conjugated antibody (Sigma Chemical Co., St. Louis, Mo.) diluted in TBST. The substrates Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used for color development.

Proteins that reacted with specific antibodies within the 2-D PAGE gel were mapped with 0.1% India ink as a secondary stain for the total protein population after detection by immunoblotting (19). Alternatively, the digoxigenin total protein/antigen double staining kit (Boehringer Mannheim, Indianapolis, Ind.) was used for antibody-reactive proteins that could not be mapped by secondary staining with India ink. Briefly, after electroblotting, the membranes were washed three times in 0.05 M K_2HPO_4 (pH 8.5). The total protein population was conjugated to digoxigenin by incubating the membrane for 1 h at room temperature in 0.05 M K_2HPO_4 (pH 8.5)–0.38 nM digoxigenin-3-*O*-methylcarbonyl-ε-amino-caproic acid *N*-hydroxysuccinimide ester–0.01% Nonidet P-40. The membranes were subsequently blocked with a solution of 3% bovine serum albumin in 0.05 M Tris-HCl (pH 7.5)–0.15 M NaCl (TBS) for 1 h and then washed with TBS. Incubation with specific antibodies was performed as described above, followed by a 1-h incubation of the membranes with mouse anti-digoxigenin-Fab fragments conjugated to alkaline phosphatase diluted 1:2,000 in TBS. The membranes were washed three times with TBS and probed with goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody. Color development for the proteins reacting to the specific anti-*M. tuberculosis* protein antibodies was performed with the substrates 4-(1,4,7,10-tetraoxadecyl)-1-naphthol and 1.8% H_2O_2. Secondary color development of the total protein population labeled with digoxigenin was performed with BCIP and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride as the substrates.

TABLE 1. Pattern of reactivity of CFPs of *M. tuberculosis* H37Rv with known MAbs and polyclonal sera

Antibody[a]	Reported corresponding antigen	Reference[e]	Dilution of antibodies
IT-1 (F23-49-7)	16 kDa[b]	28	1:2,000
IT-3 (SA-12)	12-kDa GroES[c]	37	1:8,000
IT-4 (F24-2-3)	16-kDa α-crystallin[c]	28	1:2,000
IT-7 (F29-29-7)	40 kDa[c]	28	1:1,000
IT-10 (F29-47-3)	21 kDa[c]	28	1:1,000
IT-12 (HYT6)	19-kDa lipoprotein[c]	3	1:50
IT-17 (D2D)	28-kDa SOD[c]	65	1:8,000
IT-20 (WTB68-A1)	14-kDa α-crystallin[c]	10	1:250
IT-23 (WTB71-H3)	38-kDa PstS[c]	10	1:250
IT-40 (HAT1)	71-kDa DnaK[c]	3	1:50
IT-41 (HAT3)	71-kDa DnaK[c]	3	1:50
IT-42 (HBT1)	82 kDa[c]	35	1:50
IT-43 (HBT3)	56 kDa[c]	35	1:50
IT-44 (HBT7)	32 kDa[c]	35	1:50
IT-45 (HBT8)	96 kDa[c]	35	1:50
IT-46 (HBT10)	40 kDa[b]	35	1:50
IT-49 (HYT27)	32–33-kDa Ag85 complex[c]	50	1:50
IT-51 (HBT2)	17 kDa[c]	35	1:50
IT-52 (HBT4)	25-kDa MPT 51[c]	35	1:50
IT-53 (HBT5)	96 kDa[c]	35	1:50
IT-56 (CBA1)	65 kDa[d]	35	1:50
IT-57 (CBA4)	82 kDa[c]	35	1:50
IT-58 (CBA5)	47 kDa[c]	35	1:50
IT-59 (F67-1)	33 kDa[c]	28	1:100
IT-61 (F116-5)	30 kDa[c]	60	1:100
IT-67 (L24.b4)	24-kDa MPT 64[c]	40	1:50
IT-69 (HBT 11)	20 kDa[c]	35	1:6
F126-2	30 kDa[c]	60	1:100
A3h4	27 kDa[c]	45	1:50
HYB 76-8	6-kDa ESAT6[c]	27	1:100
Anti-MPT 32	50-kDa MPT 32[c]	39	1:100
Anti-MPT 46	10-kDa MPT 46[c]	39	1:100
Anti-MPT 53	15-kDa MPT 53[c]	39	1:100
Anti-MPT 57	12-kDa GroES[c]	39	1:100
Anti-MPT 63-K64	18-kDa MPT 63[c]	39	1:200

[a] Original designations for the WHO Scientific Working Groups on Immunology of Leprosy and Immunology of Tuberculosis cataloged MAbs are given in parentheses.

[b] Identified in the CFPs of *M. tuberculosis* H37Rv used in this study by 1-D Western blot analysis.

[c] Identified in the CFPs of *M. tuberculosis* H37Rv used in this study by 1-D and 2-D Western blot analysis.

[d] Not detected in the CFPs of *M. tuberculosis* H37Rv used in this study.

[e] References are for the MAbs or polyclonal sera.

Amino acid sequence analysis. To obtain the N-terminal amino acid sequence for selected proteins, CFPs (200 μg) were resolved by 2-D PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Milford, Mass.) by electroblotting at 50 V for 1 h, with cyclohexylaminopropanesulfonic acid buffer with 10% methanol. The membrane was stained with 0.1% Coomassie brilliant blue in 10% acetic acid and destained with a solution of 50% methanol–10% acetic acid. Immobilized proteins were subjected to automated Edman degradation on a gas phase sequencer (22) equipped with a continuous-flow reactor (54). The phenylthiohydantoin amino acid derivatives were identified by on-line reversed-phase chromatography (54).

LC-MS-MS analysis. Selected CFPs were subjected to LC-MS-MS to determine the sequence of internal peptide fragments. CFPs (200 μg) were resolved by 2-D PAGE, and the gel was stained with 0.1% Coomassie brilliant blue and destained as described for proteins immobilized to polyvinylidene difluoride membranes. The protein of interest was excised from the gel, washed several times with distilled water to remove residual acetic acid, and subjected to an in-gel proteolytic digestion with trypsin (23). Peptides were eluted from the

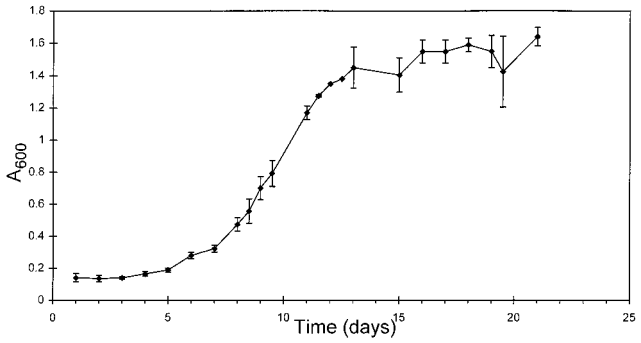

FIG. 1. Growth curve of *M. tuberculosis* H37Rv. Data points show the mean of three cultures grown simultaneously in GAS medium with 0.05% Tween 80. Error bars represent standard deviations.

acrylamide and separated by C_{18} capillary reversed-phase chromatography (23). The microcapillary reversed-phase effluent was introduced directly into a Finnigan-MAT (San Jose, Calif.) TSQ-700 triple-sector quadrupole mass spectrometer. MS and analysis of the data were performed as described by Stahl et al. (57). MS was performed by K. M. Swiderek, Beckman Research Institute of the City of Hope, Duarte, Calif.

RESULTS

Identification and 2-D PAGE mapping of known CFPs of *M. tuberculosis* H37Rv. Through the efforts of the World Health Organization (WHO) Scientific Working Groups on the Immunology of Leprosy and Immunology of Tuberculosis, an extensive collection of MAbs against mycobacterial proteins has been established (17, 26). This library, as well as MAbs and polyclonal sera not included in the WHO collections, allowed the identification and mapping of known mycobacterial proteins in the culture filtrate of *M. tuberculosis* (Table 1). All 35 antibodies tested, except IT-56, were reactive in a 1-D Western blot format against day 14 CFPs from *M. tuberculosis* H37Rv (Table 1). The MAb IT-56 is specific for the 65-kDa *M. tuberculosis* GroEL homolog (35), a protein primarily associated with the cytosol (53). Three additional MAbs (CS43, CS44, and CS45) specific for the 65-kDa GroEL were tested and yielded no reactivity with the CFPs. Additionally, the MAb IT-7 reacted with a 14-kDa CFP and not a 40-kDa CFP as previously reported (28).

The lack of detectable amounts of the 65-kDa GroEL homolog, a marker for autolysis (5, 39), in the 2-week culture filtrate preparations used for these studies was contradictory to the observations of Andersen et al. (5). Growth curves generated for *M. tuberculosis* H37Rv demonstrated that the CFPs were harvested during the late log growth phase (Fig. 1).

By using 2-D Western blot analysis coupled with secondary staining (either India ink or digoxigenin total protein/antigen double staining), 32 of the antibodies were mapped to proteins within the 2-D PAGE profile (Table 1; Fig. 2). However, two antibodies (IT-1 and IT-46), which were reactive by 1-D Western blot analysis, failed to detect any CFPs resolved by 2-D PAGE (Table 1; Fig. 2). This lack of reactivity by 2-D Western blot analysis presumably was due to the absence of linear epitopes exposed by the denaturing conditions used to resolve molecules for conventional Western blot analyses (59).

Most of the antibodies recognized a single protein. However, several (IT-3, IT-4, IT-7, IT-20, IT-23, IT-41, IT-42, IT-44, IT-49, IT-57, IT-58, IT-61, and MPT 32) reacted with multiple proteins (Fig. 2). Five of these (IT-23, IT-42, IT-44, IT-57, and IT-58) reacted with protein clusters centered at 36, 85, 31, 85, and 50 kDa, respectively. It is noteworthy that the proteins in each of these clusters migrated within a narrow pI range, suggesting that the antibodies were reacting with multiple isoforms of their respective proteins. For the protein cluster at 85 kDa, detected by IT-57, the most dominant component of this cluster was also recognized by IT-42.

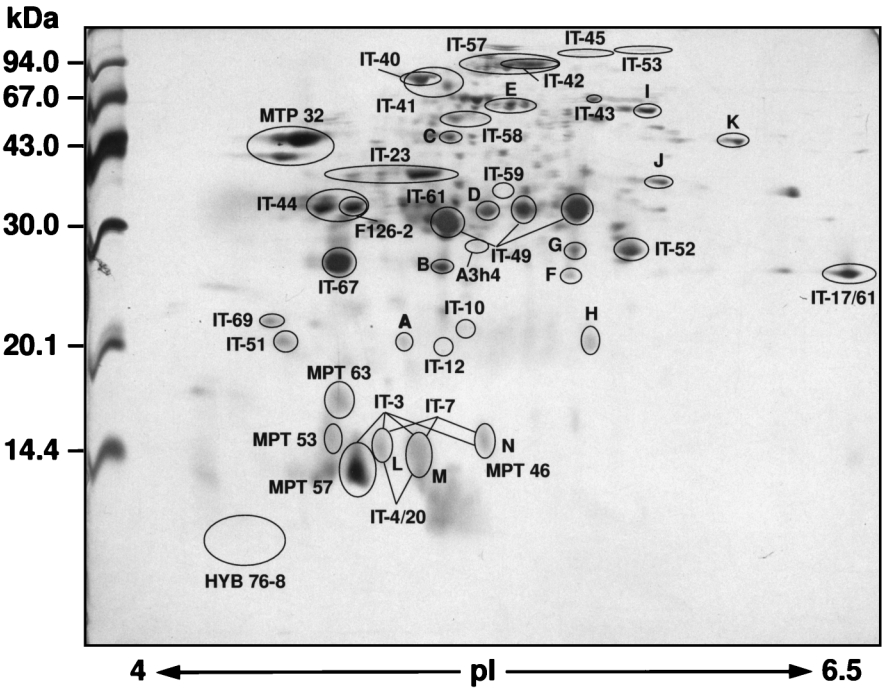

FIG. 2. 2-D PAGE of CFPs from *M. tuberculosis* H37Rv. Known proteins are designated by the name of the MAb or polyclonal sera with which they reacted. Unidentified proteins selected for N-terminal amino acid sequencing are labeled A to N.

TABLE 2. N-terminal amino acid sequences or internal peptide fragments identified by LC-MS-MS of selected CFPs of *M. tuberculosis* $H_{37}Rv$

Protein	N-terminal amino acid sequence	Internal peptides mapped[b]	$(M + H)^+$		Homology[c]
			Predicted	Observed	
A	None[a]				
B	APPSCAGLD/GCTV				
C	XXAVXVT				
D	FSRPGLPVEYLQVPSP				*M. tuberculosis* Ag85A and Ag85B
E	TEKTPDDVFKLADDEKVEYVD				*M. tuberculosis* glutamine synthetase
F	XPVM/LVXPGXEXXQDN				*M. tuberculosis* cosmid MTCY1A11
G	None				
H	None				
I	XVYDVIMLTAGP				*Eubacterium* sp. strain VPI 12708 α-hydroxysteroid dehydrogenase
J	None				
K	None				
L	RLMRLEDEMKEGRYEVR				Truncated form of 16-kDa α-crystallin homolog
M	RLMRLEDEMKEGRYEVRAELPGVD				Truncated form of 16-kDa α-crystallin homolog
N	TDSEKSATIKVTDACFA				MPT 46 (thioredoxin)
IT-43	None				
IT-42/57	None				
IT-58	K/NVIRIXGXTD				
F126-2	None				
IT-42		FAPLNSWPDNASLDK (129–143)	1,675.8	1,676.4	KatG
		EATWLGDER (201–209)	1,077.1	1,075.7	KatG
		DAITSGIEVVWTNTPTK (310–326)	1,833.0	1,834.4	KatG
		SPAGAWQYTAK (345–355)	1,180.3	1,179.2	KatG
		DGAGAGTIPDPFGGPGR (356–372)	1,542.6	1,542.1	KatG
		WLEHPEELADEFAK (396–409)	1,714.9	1,716.0	KatG
		TLEEIQESFNSAAPGNIK (519–536)	1,949.1	1,950.0	KatG
		AAGHNITVPFTPGR (557–570)	1,438.6	1,438.7	KatG
		TDASQEQTDVESFAVLEPK (571–589)	2,095.2	2,096.1	KatG
		GNPLPAEYMLLDK (600–612)	1,461.7	1,460.7	KatG
		ANLLTLSAPEMTVLVGGLR (613–631)	1,956.4	1,955.9	KatG
		VDLVFGSNSELR (693–704)	1,336.5	1,334.7	KatG
		ALVEVYGADDAQPK (705–718)	1,476.7	1,476.8	KatG
		FVQDFVAAWDK (719–729)	1,326.5	1,326.1	KatG

[a] None, proteins were refractory to sequencing by Edman degradation.
[b] Assignment of peptides was based on the sequence reported by Zhang et al. (67).
[c] Homology was based on the molecular ion $(M + H)^+$ and fragment ions obtained by MS-MS.

Polyclonal serum against MPT 32 recognized 45- and 42-kDa proteins with relatively similar pIs. Previously, while defining sites of glycosylation on MPT 32 (15), we observed that this protein was prone to autoproteolysis and formed a 42-kDa product (unreported results). Thus, the 42-kDa protein detected with the anti-MPT 32 serum is apparently a breakdown product of the 45-kDa MPT 32 glycoprotein.

MAb IT-49, specific for the antigen 85 (Ag85) complex (50), clearly identified the three gene products (Ag85A, Ag85B, and Ag85C) of this complex. Furthermore, the 2-D pattern observed for this complex was similar to that described by Nagai et al. (39). The region of most intense antibody cross-reactivity was observed below 16 kDa. The most prominent protein in this region reacted with a MAb (IT-3) and polyclonal sera (anti-MPT 57) specific for the 14 kDa GroES homolog (37, 39). Several adjacent proteins at approximately 14 kDa were also recognized by IT-3. Interestingly, various members of this protein cluster reacted with anti-MPT 46 polyclonal serum and the MAbs IT-4, IT-7, and IT-20.

N-terminal amino acid sequencing of selected CFPs. The N-terminal amino acid sequences or complete gene sequences and functions of several of the CFPs of *M. tuberculosis*, mapped with the available antibodies, are known (66). However, such information is lacking for the proteins that reacted with IT-42, IT-43, IT-44, IT-45, IT-51, IT-53, IT-57, IT-59, IT-69, and F126-6, as well as several unidentified abundant proteins, as observed by silver staining. Of these, those reacting with IT-42/IT-57, IT-43, IT-58, and F126-2, as well as the proteins labeled A to K; were selected and subjected to N-terminal amino acid sequencing (Fig. 2; Table 2). N-group analysis was also performed on the three low-molecular-weight proteins (labeled L to N) that cross-reacted with IT-3, IT-4, IT-7, IT-20, and anti-MPT 46 (Fig. 2; Table 2).

Three of these proteins were found to correspond to previously defined products. The N-terminal amino acid sequence of the protein labeled D was identical to that of Ag85B and Ag85C. This result was unexpected, given that the IT-49 MAb failed to detect this protein, and N-terminal amino acid analysis confirmed that the proteins reacting with IT-49 were members of the Ag85 complex. A second protein, labeled E, yielded an N-terminal amino acid sequence identical to that of the glutamine synthetase recently characterized by Harth et al. (20), and the protein labeled N was confirmed to be the 15-kDa thioredoxin (62). However, five of the proteins subjected

to N-terminal analysis appeared to be novel. Three of these, those labeled B, C, and IT-58, did not demonstrate significant homology to any known mycobacterial or prokaryotic sequences. However, the protein labeled I possessed an N-terminal sequence with 72% identity to the amino terminus of an α-hydroxysteroid dehydrogenase from a *Eubacterium* sp. (12), and the protein labeled F was homologous to a segment of the deduced amino acid sequence for an open reading frame identified in the *M. tuberculosis* cosmid MTCY1A11 (44). Identical N-terminal amino acid sequences were obtained for the proteins labeled L and M. Moreover, this data demonstrated that these two products were truncated forms of the 16-kDa α-crystallin homolog (33), each with an N-terminal amino acid corresponding to residue 38 of the full-length protein. Repeated attempts to sequence the proteins labeled as A, G, H, J, K, IT-43, IT-42/IT-57, and F126-2 were unsuccessful.

Previously, we demonstrated that a high-molecular-weight fraction of CFP of *M. tuberculosis* reacted with a preponderance of sera from tuberculosis patients (29). Moreover, this fraction was distinguished from other protein fractions in that it possessed the product that reacted with MAb IT-57. In view of this information, the protein cluster defined by IT-42 and IT-57 was excised from a 2-D polyacrylamide gel and digested with trypsin and the resulting peptides were analyzed by LC-MS-MS. A digest of the portion of this protein cluster that reacted with both IT-42 and IT-57 (Fig. 2) yielded 14 peptides with molecular masses and fragmentation patterns consistent with those predicted for trypsin-generated fragments of the *M. tuberculosis* KatG catalase/peroxidase (Table 2). Tryptic digestion and LC-MS-MS analysis of the proteins that reacted only with MAb IT-57 (Fig. 2) did not reveal homology to any known mycobacterial proteins. Therefore, it appears that the protein cluster defined by MAbs IT-42 and IT-57 is composed of the KatG and a novel unidentified protein.

Computer-aided analysis of the 2-D PAGE CFP profile of *M. tuberculosis* H37Rv. Computer-aided analysis of the 2-D polyacrylamide gel of *M. tuberculosis* H37Rv was performed to determine the total number of protein spots in this fraction and to develop a detailed numerical map. Three separate lots of CFPs of *M. tuberculosis* H37Rv were pooled and resolved by 2-D PAGE. The silver-stained gel was digitized, and the data were analyzed with the Microscan 1000 2-D gel analysis software. In all, 205 protein spots were detected for *M. tuberculosis* H37Rv (Fig. 3). Individual proteins were numbered sequentially from acidic to basic pI and by descending molecular weight.

As described above, the mapping of specific CFPs with monoclonal antibodies clearly indicated that multiple isoforms of some CFPs are observed by 2-D PAGE. Thus, each protein spot depicted in Fig. 3 does not represent an individual gene product. Without the availability of MAbs for all of the CFPs or of N-group or LC-MS-MS analyses of each spot, it is not possible to determine the total number of gene products represented in the culture filtrate used for these studies. Nevertheless, of the 205 protein spots observed in Fig. 3, 31 (spots 7, 10, 11, 12, 14, 22, 25, 26, 29, 31, 37, 38, 46, 52, 65, 66, 76, 79, 81, 82, 87, 92, 93, 102, 119, 149, 151, 155, 170, 175, and 201) were found to represent 24 gene products by virtue of their reactivity with MAbs or polyclonal antibodies (Table 3). N-terminal amino acid sequencing or LC-MS-MS demonstrated that 13 spots (spots 45, 60, 74, 80, 97, 101, 103, 113, 114, 120, 124, 144, and 176) represented 10 additional gene products. In total, 34 individual gene products were mapped to 44 protein spots characterized by immunoreactivity or molecular methods.

DISCUSSION

In comparison to the proteins of other subcellular fractions of *M. tuberculosis* (cell wall, cell membrane, and cytoplasm), the CFPs, as a family, are well defined in terms of function, immunogenicity, and composition (1). For instance, Nagai et al. (39) identified 12 of the most abundant excreted proteins of *M. tuberculosis*, and others subsequently defined the various biological activities of these molecules (1) or novel products (56). Furthermore, an additional 23 MAbs produced by various laboratories have been shown to react with individual CFPs (Table 1). Our 2-D PAGE analysis demonstrated that 205 protein spots are present in the culture filtrate of *M. tuberculosis* H37Rv and that of this total, only 34 individual proteins could be identified with a collection of MAbs and polyclonal sera or by molecular characterization (Table 3). Thus, an overall understanding of the molecular composition of CFPs is lacking. This is underscored by the fact that amino acid or complete gene sequences were previously known for only 18 of the proteins mapped. We have now expanded this number by providing sequence information for six previously undefined culture filtrate proteins. Many of the proteins submitted for N-group analysis were refractory to Edman degradation, and thus the continuation of this type of biochemical characterization on a broader spectrum of CFPs is not feasible. Nonetheless, completion of the *M. tuberculosis* genome sequencing project in the near future will provide information about additional excreted proteins and allow MS data of CFPs to be rapidly matched to specific genes.

Mapping of known proteins. In mapping of known proteins, it was noted that several of the antibodies recognized more than a single spot and that several of these appeared to be reacting to multiple isoforms of the same protein or recognizing more than a single gene product. While most of the antibodies yielded clean and definitive results, several different MAbs previously assigned to well-characterized proteins exhibited cross-reactivity with a cluster of four proteins in the 14- to 15-kDa range. The most dominant and acidic protein of this cluster (spot 38 in Fig. 3 and Table 3) reacted with MAb IT-3 and anti-MPT 57 polyclonal serum, both of which are specific for the 14-kDa GroEs homolog (37, 39). Thus, we concluded that protein 38 is the 14-kDa GroES homolog. The IT-3 antibody also detected three adjacent protein spots (spots 45, 60, and 97); of these, proteins 45 and 60 were additionally recognized by MAbs IT-4 and IT-20, specific for the 16-kDa α-crystallin (10, 28, 33). However, protein 97 reacted specifically with anti-MPT 46 polyclonal serum, indicating that it was the previously defined thioredoxin of *M. tuberculosis* (62). N-group analysis of these three proteins confirmed that protein 97 was the thioredoxin and that proteins 45 and 60 were truncated forms of the α-crystallin homolog. Both of the last two proteins lacked the first 37 amino acids of the full-length product. The predicted size of *M. tuberculosis* α-crystallin minus the first 37 amino acids is 12,062 Da. Further proteolytic cleavage at the C terminus of one of these truncated molecules or some other form of posttranslational modification may account for the observed difference in the 2-D migration of proteins 45 and 60.

N-group analysis and the Ag85 complex. To add further molecular definition to the CFPs of *M. tuberculosis*, a number of abundant products, as observed by 2-D PAGE, were subjected to N-group analysis. One of these, protein 103, which migrated between Ag85B (protein 81) and Ag85C (protein 119), yielded 16 amino acid residues (FSRPGLPVEYLQV PSP) identical to the amino terminus of mature Ag85A (8) and Ag85B (36) and differed from that of Ag85C (11) by a single amino acid residue at position 15. From this analysis, it ap-

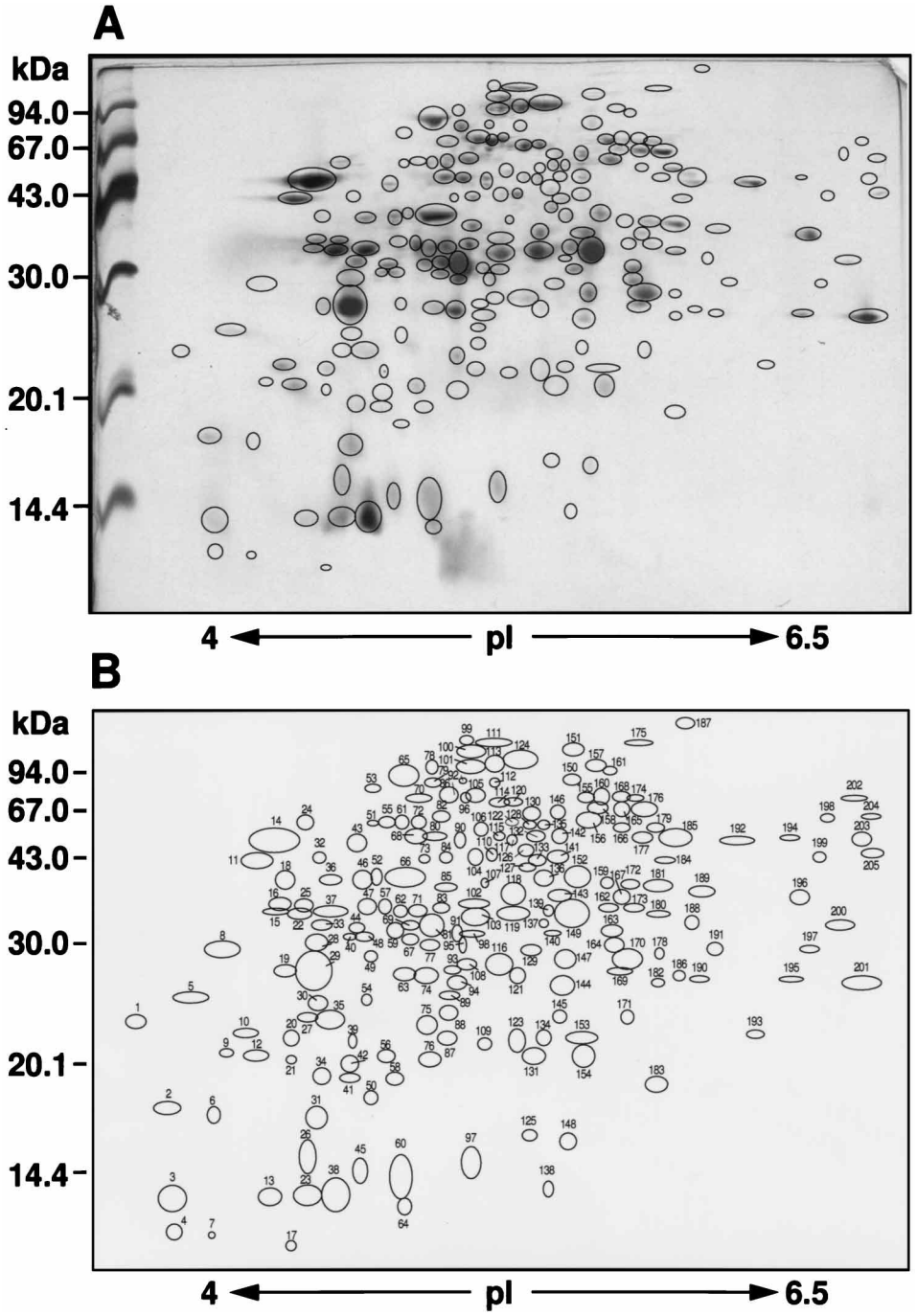

FIG. 3. 2-D PAGE map of CFPs of *M. tuberculosis* H37Rv. (A) Silver nitrate-stained 2-D polyacrylamide gel of CFPs of *M. tuberculosis* H37Rv overlaid with the digitized image of protein spots detected with the MicroScan 1000 2-D gel analysis software. (B) Digitized image of the 2-D gel of CFPs of *M. tuberculosis* H37Rv. Reference numbers of individual protein spots correspond to those listed in Table 3.

peared that protein 103 was an isomer of Ag85A or Ag85B. However, the increased molecular weight of this protein over that of Ag85B and its shift in pI in relation to Ag85A suggested that it may have resulted from posttranslational modifications. This is unlikely in light of a recent report by Harth et al. (21), which indicated that members of the Ag85 complex lacked posttranslational modifications. Alternatively, the complete lack of reactivity of protein 103 with the Ag85 complex-specific MAb (IT-49) and the homology of its N-terminus suggested that this product may be a fourth member of the Ag85 complex. Others have reported additional protein bands associated with the Ag85 complex by isoelectric focusing (11), but direct evidence to support the existence of a fourth member of this complex is lacking. Further sequencing of internal fragments will ultimately determine the true origin of protein 103.

Confirmation of a novel 25-kDa protein. N-group analysis of a 25-kDa protein with a pI of 5.34 (protein 144) resulted in a sequence (XPVM/LVXPGXEXXQDN) homologous to an in-

TABLE 3. Summary of protein spots detected by computer-aided analysis of silver nitrate-stained 2-D gels

Protein no.[a]	Mol mass (kDa)[b]	pI[c]	Antibody reactivity	Function/designation	N-terminal sequence[d]	Reference(s)[h]
7	11.75	3.52	HYB 76-8	ESAT 6	TEQQWNFAGI[e]	56
10	21.63	4.14	IT-69			
11	38.90	4.31	Anti-MPT 32	MPT 32	DPEPAPPVPT[e]	15
12	20.07	4.31	IT-51			
14	42.17	4.51	Anti-MPT 32	MPT 32	DPEPAPPVPT[e]	15
22	31.44	4.75	IT-44			
25	32.55	4.79	IT-44			
26	15.67	4.79	Anti-MPT 53	MPT 53	DECIQ[e]	39
29	26.15	4.83	IT-67	MPT 64	APKTYCEELK[e]	39
31	16.88	4.84	Anti-MPT 63	MPT 63	AYPIT[e,g]	39
37	31.44	4.93	IT-44/F126-2			
38	14.45	4.93	Anti-MPT 57/IT-3	GroES homolog/MPT 57	MAKVNIKPLE[e]	7
45	14.96	5.02	IT-3/4/7/20	Truncated α-crystallin	*RLMRLEDEMK*	
46	35.48	5.03	IT-23	PstS	MKIRLHTLLA[f]	2
52	35.89	5.06	IT-23	PstS	MKIRLHTLLA[f]	2
56	20.18	5.07		A		
60	14.54	5.09	IT-3/4/7/20	Truncated α-crystallin	*RLMRLEDEMK*	62
65	72.86	5.09	IT-40/IT-41	DnaK homolog	MARAVGIDLG[f]	32
66	35.69	5.09	IT-23	PstS	MKIRLHTLLA[f]	2
74	25.56	5.10		B	*APPSCAGLD/GC*	
76	19.61	5.10	IT-12	19-kDa lipoprotein	MKRGLTVAVA[f]	6
79	66.83	5.10	IT-41	DnaK homolog	MARAVGIDLG[f]	32
80	42.17	5.10		C	*XXAVXVT*	
81	29.85	5.10	IT-49/IT-61	Ag85 B/MPT 59	FSRPGLPVEY[e,g]	36
82	49.55	5.10	IT-58		*K/NVIRIXGXTD*	
87	20.89	5.11	IT-10			
92	69.98	5.11	IT-41	DnaK homolog	MARAVGIDLG[f]	32
93	26.15	5.11	A3h4			
97	15.22	5.11	Anti-MPT 46/IT-3/7	Thioredoxin/MPT 46	TDSEKSATIK[e,g]	62
101	82.22	5.12	IT-57			
102	32.73	5.12	IT-59			
103	31.08	5.12		D: Ag85 homolog	*FSRPGLPVEY*	
113	85.11	5.14	IT-57			
114	55.59	5.14		E: Glutamine synthetase	TEKTPDDVFK[e,g]	20
119	31.08	5.17	IT-49	Ag85 C/MPT 45	FSRPGLPVEY[e,g]	11
120	55.59	5.17		E: Glutamine synthetase	TEKTPDDVFK[e,g]	20
124	85.11	5.19	IT-42/IT-57	KatG	MPEQHPPITE[f]	65
144	24.97	5.34		F	*XPVM/LVXPGXE*	
147	26.92	5.37		G		
149	31.44	5.38	IT-49	Ag85A/MPT 44	FSRPGLPVEY[e,g]	8
151	94.41	5.40	IT-45			
154	20.07	5.47		H		
155	58.88	5.50	IT-43			
170	26.92	5.91	IT-52	MPT 51	APYENLMVPS[e,g]	39, 64
175	98.86	6.08	IT-53			
176	52.48	6.18		I	*XVYDVIMLTA*	
181	34.47	6.42		J		
192	40.74	8.39		K		
201	24.83	<10	IT-17/IT-61	SOD/MPT 58	MAEYTLPDLD[f]	68

[a] Reference numbers correspond to those in Fig. 3.
[b] Molecular masses were calculated from a standard curve generated by using the molecular masses of the protein standards (Pharmacia) and their relative mobilities within the gel.
[c] The isoelectric point for each protein was determined from a standard curve generated with the reported isoelectric points for MPT 32 (4.5), MPT 57 (4.9), MPT 46 (5.1), Ag85C (5.15), Ag85A (5.4), and MPT 51 (5.9).
[d] N-terminal sequences obtained in this study are in italics. The first 10 amino acids of the N-terminal sequence are shown.
[e] Reported observed N-terminal sequence of mature protein.
[f] Reported N-terminal sequence deduced from nucleotide sequence.
[g] Sequence confirmed in this study.
[h] References are for N-terminal sequences.

ternal fragment (DPVLVFPGMEIRQDN) of a product encoded by open reading frame 28c on the *M. tuberculosis* cosmid MTCY1A11 (44). Analysis of the deduced amino acid sequence revealed a signal peptidase I consensus sequence (Ala-Xaa-Ala) (31) and what appeared to be a signal peptide preceding the N terminus of the 25-kDa protein sequenced by us. Although the function of this protein is unknown, this analysis has confirmed the validity of open reading frame MTCY 11A11.28c.

N-terminal sequencing of four additional CFPs. Analysis of a 56-kDa protein cluster (proteins 114 and 120) identified it as the previously defined glutamine synthetase. A third protein (protein 176) possessed an N-terminal sequence that demonstrated 72% identity to the N-terminal sequence of a 42-kDa

α-hydroxysteroid dehydrogenase of *Eubacterium* sp. strain VPI 12708 (12). Three additional proteins (proteins 74, 80, and 82) for which N-terminal sequencing was obtained showed no significant homology to known peptides. For these proteins and those that were refractory to N-group analysis, more advanced methods of protein sequencing such as LC-MS-MS will circumvent the problems associated with N-blocked proteins and provide extended sequence data (57).

LC-MS-MS analysis of the seroreactive 83- to 85-kDa protein cluster. The identity of the protein cluster (proteins 101, 113, and 124) which was recognized by MAbs IT-42 and IT-57 was a primary objective of this study. These proteins migrated in a molecular mass range of 82 to 85 kDa and a pI range of 5.12 to 5.19. Previous work conducted in collaboration with Suman Laal identified a culture filtrate product of approximately 88 kDa that reacted with 70% of the sera from tuberculosis patients and demonstrated a specificity of 100% (29). Subsequent analyses with the 2-D maps generated in this study coupled with 2-D Western blot analysis indicated that these dominant humoral antigens were the same as those that reacted with IT-57 and IT-42 (49). Repeated attempts to obtain N-terminal sequences for the proteins of this complex were unsuccessful. However, the use of LC-MS-MS demonstrated that protein 124 was the KatG catalase/peroxidase (13, 67). This protein was previously designated MPT 35 by Nagai et al. (39). A similar analysis of proteins 101 and 103 of this cluster was inconclusive. However, the apparent reactivity of these two proteins with only MAb IT-57 and not IT-42 suggests that they may be novel products that share an epitope with KatG.

Growth phase and composition of *M. tuberculosis* culture filtrate. Several studies have noted that the incubation time of *M. tuberculosis* cultures has a dramatic effect on the profile of proteins released into the culture supernatant by the tubercle bacilli (5, 39). In particular, the work of Andersen et al. (5) demonstrates that a small, well-defined set of proteins is actively excreted during the first 3 days of incubation and that a gradual secretion of cell wall proteins occurs during the logarithmic growth phase. Furthermore, the release of cytoplasmic proteins, as monitored by the presence of isocitrate dehydrogenase and the 65-kDa GroEL homolog, is not observed until the end of the logarithmic growth phase. The present study was done with culture filtrate preparations harvested during the late logarithmic growth phase of *M. tuberculosis*. However, Western blot analysis of these preparations with MAbs IT-56, CS43, CS44, and CS45 as probes demonstrated the absence of the GroEL homolog. Other *M. tuberculosis* proteins such as the 34-kDa protein, reacting with MAb F126-2, and the 70-kDa DnaK homolog have also been associated with the release of somatic proteins (63), and both of these products were observed in significant quantities in our culture filtrate preparations. Wiker et al. (63) also observed "barely detectable" levels of the 65-kDa GroEL homolog in their late-logarithmic-phase culture filtrate. Therefore, we conclude that the late-logarithmic-phase harvest of *M. tuberculosis* culture filtrate, as performed in the present study, resulted in a fraction containing a limited number of cytoplasmic constituents, as well as actively excreted and cell wall proteins released during logarithmic growth. The lack of detectable amounts of the 65-kDa GroEL homolog may be explained by differences in the culture media used, namely, the lack of Tween 80 in the GAS medium. It should also be noted that we observed late-logarithmic-phase growth from days 10 to 14 whereas this growth phase was achieved by day 6 in the study by Andersen et al. (5).

The generation of a 2-D protein map of the culture filtrate of *M. tuberculosis* H37Rv now provides a baseline for the evaluation of the culture filtrates of other type strains and well-defined clinical isolates (42). Such an analysis is warranted, given the recent observations that several *M. tuberculosis* isolates lack the gene encoding MPT 40 (61), and the loss of KatG activity in many isoniazid-resistant strains of *M. tuberculosis* is associated with the concurrent overexpression of AhpC (52). This type of broad survey of virulent *M. tuberculosis* strains may lead to the identification of novel virulence factors or immunologically relevant proteins and add to our understanding of the physiology of *M. tuberculosis* as a whole. Furthermore, the continued molecular characterization of the proteins which constitute the various subcellular fractions of *M. tuberculosis* by 2-D PAGE coupled with powerful techniques such as LC-MS-MS will supplement the wealth of information that is now being derived from the *M. tuberculosis* genome-sequencing project.

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