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Review

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What Can Proteomics Tell Us about Tuberculosis?

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Tuberculosis (TB) is an infectious disease transmitted by aerosol droplets and characterized by forming granulomatous lesions. Although the number of people infected in the population is high, the vast majority does not exhibit symptoms of active disease and only 5–10% develop the disease after a latent period that can vary from weeks to years. The bases of the immune response for this resistance are unknown, but it depends on a complex interaction between the environment, the agent, and the host. The analysis of cellular components of *M. tuberculosis* shows important host-pathogen interactions, metabolic pathways, virulence mechanisms, and mechanisms of adaptation to the environment. However, the *M. tuberculosis* proteome still remains largely uncharacterized in terms of virulence and pathogenesis. Here, we summarize some of the major proteomic studies performed to scrutinize all the mycobacterial components.

Keywords: *M. tuberculosis*, *M. bovis*, cellular components, culture filtrate, proteome

Introduction

Tuberculosis (TB) is the second leading cause of death from a single infectious agent, after the immunodeficiency virus (HIV). In 2013, 9.0 million people developed the disease and 1.5 million died, and 25% of them were HIV positive [106]. In fact, TB is the leading killer of people infected with HIV, causing one fifth of all deaths in this population. Most deaths due to TB occur in men; however, TB is the third most important cause of death in women; half of the deaths in the HIV-positive group are woman. Six percent of all cases are children under 15, and 8% of them die. Currently, multidrug-resistant TB is present in almost all countries surveyed [106].

Tuberculosis is caused by *Mycobacterium tuberculosis*, a member of the *M. tuberculosis* complex, which also includes *M. bovis*, *M. microti*, *M. caprae*, *M. canettii*, and *M. africanum* [26], most of them capable of causing disease in humans. Typically, TB affects the lungs and the lymph nodes, but it can actually affect any organ in the body [53]. Infection establishes in approximately one third of the individuals exposed to the tuberculous bacillus; however, only about 10% ever develop the disease [16, 45, 76].

There are quite a few reviews in the literature about studies analyzing the complex immune response towards *M. tuberculosis* in infected individuals, but only a few explore the interaction between the host cells and the bacilli [25, 68, 74, 81, 85]. There are also studies that review the most important mycobacterial components during infection [29, 33, 34, 37]. However, the proteome of *M. tuberculosis* remains unclear, especially in terms of virulence and pathogenesis [56], in spite of the increasing interest in detecting mycobacterial antigens related to the immune responses in skin testing. Before the 90's, many mycobacterial proteins were identified using methods based essentially on biochemical fractionation and on immunological screening with monoclonal antibodies in patient sera. At that time, important proteins such as the Ag85 complex, MPB64, MPB70, and some cytoplasmic proteins like DnaK, GroEl, and SodA were identified [18, 112].

In the last two decades, there has been increasing interest in studying the proteome of *M. tuberculosis*. Classical studies involve two-dimensional electrophoresis (2-DE), in which proteins are separated according to charge and

molecular weight. This method resolves typically between 1,500 and 3,000 spots [105]. Protein spots can be visualized with silver or Coomassie staining or by fluorescent dyes. Every spot is then isolated and digested with trypsin to produce 10 mer peptides, which are then subjected to techniques like mass spectrometry (MS), tandem MS (MS/MS); matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). Nagai *et al.* [67] were able to purify and partially characterize 12 different proteins from culture filtrate of *M. tuberculosis* using one- and two-dimensional electrophoresis to monitor the separation of individual proteins during various chromatographic procedures. Two-dimensional liquid chromatography (LC-MS/MS) and the isotope-coded affinity tag technology (ICAT) have also been reported in proteomic studies [10, 40]. Other authors combined 2-DE, western blotting, N-terminal amino acid sequencing, and liquid chromatography-mass spectrometry (LC-MS-MS) to identify 205 spots and map 32 N-terminal sequences [96]. These original studies had led to the creation of 2-DE maps, but it was until the description of the complete genome sequence of *M. tuberculosis* that proteomic analysis really helped to understand this pathogen. In this review we summarize the state of art about the proteomics of *M. tuberculosis.*

Cellular Components

The analysis of cellular components of *M. tuberculosis* shows important host-pathogen interactions, metabolic pathways, virulence mechanisms, and mechanisms of adaptation to the environment. For example, the analysis of culture filtrates allows the identification of proteins secreted by the bacilli, potential markers of virulence, or immunogenic antigens for the development of new vaccines and diagnostic tools. Cell wall and membrane proteins are involved in the resistance of the pathogen to chemical injury and play a fundamental role in host-pathogen interactions; therefore, the study of the cell wall proteins can be useful in the development of new drugs. Some of the proteins identified in the studies described below are listed in Table 1.

Membrane Proteins

Initial interactions between *M. tuberculosis* and the host mark the pathway of infection and the subsequent immune host-response that defines disease outcome. These interactions include many important membrane surface enzyme/transporters involved in intercellular multiplication and bacterial response to host microbicidal processes [17]. Membrane proteins are active in cell-to-cell interactions, ion transport, and cell signaling. Thus, more than half of all membrane proteins are predicted to be pharmaceutical targets [86]. Many proteins related to *M. tuberculosis* pathogenicity are surface proteins involved in lipid metabolism and transport across the membrane [34]. Identification of these proteins can help to better understand the bacterial mechanisms of virulence and help in the discovery of new drug targets. For example, some lipoproteins and glycolipoproteins (19 kDa protein, LprG, PstS1) interact with TLR2 receptors and modulate host immune responses affecting antigen presentation function of macrophages [38, 39]. In order to gain access through different tissues, bacteria have evolved a wide range of molecules to enable them to bind to select host molecules like proteoglycans, fibronectin, and laminin. Some of these molecules are the proteins of antigen 85 complex, Apa, Wag22, HbhA, and others [25] (Table 1).

Unfortunately, membrane protein characterization is difficult to accomplish, as the proteins are molecules composed of both hydrophobic and hydrophilic regions, so no single solvent or combination of solvents can solubilize all membrane proteins. Two-dimensional technology can underestimate the number of membrane proteins and membrane-associated proteins because of their low solubility. For that reason, various authors have changed the traditional 2-DE approach with 1-D SDS-PAGE technology. Gu *et al.* [43] were able to identify 739 membrane and membrane-associated proteins. They first obtained the membrane subcellular fraction of *M. tuberculosis* through a differential centrifugation method. Then, they separated this fraction on a 1-D SDS gel and treated each gel slide with trypsin, and then the peptides digested were analyzed by high-resolution microcapillary LC separation prior to a highly sensitive nanospray-MS/MS analysis. Very hydrophobic proteins, even those with 15 trans-membrane helices, were detected with this methodology.

Studies of membrane proteins have shown that the plasma membrane is easily contaminated by cytosolic and secreted proteins, which could be part of protein complexes. Xiong *et al.* [108] proposed a method to remove cytosolic proteins. It consisted of treating the plasma membrane with 5 M urea and a high pH carbonate solution, followed by ultracentrifugation. With this method, membrane proteins, together with the phospholipid bilayer, aggregate to form a pellet, whereas proteins weakly associated or bound to

Table 1. Description of some proteins identified in the studies described in this review.

Category	Gene name	Rv number	Cellular localization	Description	Function	Identified by [a]
Lipid metabolism	*kasB*	Rv2246	Membrane and cell wall fractions	3-Oxoacyl-[acyl-carrier protein] synthase 2 KasB	Involved in fatty acid biosynthesis (mycolic acids synthesis) and pathogenesis [7]. Phosphorylation of this protein regulates virulence in *M. tuberculosis* [100].	[43, 58, 108]
Lipoproteins	*pstS1*	Rv0934	Membrane and cell wall fractions	Periplasmic phosphate-binding lipoprotein PstS1 (PBP-1)	38 kDa phosphate-binding glycoprotein involved in active transport of inorganic phosphate across the membrane. Immunodominant antigen that interacts with the macrophage mannose receptor and mediates phagocytosis [27].	[41, 59, 63, 82, 84, 94, 108]
	lpqH, 19 kDa protein	Rv3763	Membrane and cell wall fractions	19 kDa lipoprotein antigen precursor LpqH	Cell-wall-associated and secreted glycoprotein; induces apoptosis in THP-1 cells and macrophages. Inhibits MHC-II expression on alveolar macrophages through TLR2 signaling [39].	[41, 43, 82, 84, 94, 108]
	lprG	Rv1411c	Membrane and cell wall fractions	27 kDa secreted glycoprotein	Inhibition of MHC-II antigen processing dependent on TLR-2 [38]. LprG binds LAM and controls its distribution in the cell envelope [90].	[41, 43, 58, 59, 94, 108]
	lprA	Rv1270c	Membrane and cell wall fractions	24 kDa cell wall associated lipoprotein	Induced expression of TNF-α, IL-10, and IL-12. Acylated LprA shows agonist activity for TLR2 [72].	[41, 43, 44, 52, 57, 58, 108]
	mpt83	Rv2873	Membrane and cell wall fractions	Cell surface lipoprotein (MPB83 in *M. bovis*)	Major antigen highly expressed by *M. bovis* and considerably less abundantly expressed by *M. tuberculosis* [104].	[9, 43, 50, 57, 62, 108]
Cell wall proteins	*ompATb*	Rv0899	Membrane and cell wall fractions	Outer membrane protein A	Pore-forming protein with properties of porin; enables bacteria to respond to reduced environmental pH [77].	[58, 59, 108]
	hbhA	Rv0475	Membrane and cell wall fractions	Heparin-binding hemaglutinin protein (Adhesin)	Required for extrapulmonary dissemination. Promotes its adherence to host tissues by binding to sulfated glycoconjugates [72].	[43, 59, 94, 108]
	mpa	Rv2115c	Membrane and cell wall fractions	Mycobacterial proteasome ATPase	Involved in proteasomal protein degradation; ATPase whose enzymatic activity is necessary to protect against reactive nitrogen intermediates [19].	[42, 65, 82]
	mpt63	Rv1926c	Culture filtrate	Immunogenic protein Mpt63 (antigen Mpt63/MPB63) (16 kDa immunoprotective extracellular protein)	One of the major secreted proteins of unknown function from *M. tuberculosis* that has been shown to have immunogenic properties and has been implicated in virulence [42].	[50, 59, 67, 82]
	mpt53	Rv2878c	Culture filtrate	Soluble secreted antigen Mpt53 precursor	MPT53 induces a vigorous antibody response and no DTH in tuberculous guinea pigs. MPT53 elicits very modest antibody responses in human TB and in bovine TB [48].	[9, 57, 58, 67, 84]
	mtc28	Rv0040c	Culture filtrate	Secreted proline-rich protein Mtc28 (proline-rich 28 kDa antigen)	It is one of the immunologically active components of the culture filtrate of *M. tuberculosis*. It elicits strong immune responses in BCG-immunized guinea pigs [61].	[21, 57, 67]
	mpt70	Rv2875	Culture filtrate	Major secreted immunogenic protein (MPB70 in *M. bovis*)	Highly soluble protein abundantly present in culture filtrate of *M. bovis* [104].	[18, 50, 63, 67, 112]

Table 1. Continued.

Category	Gene name	Rv number	Cellular localization	Description	Function	Identified by [a]
Intermediary metabolism	*sahH*	Rv3248c	Cytosol and membrane fractions	Probable adenosylhomocysteinase SahH ($_S$-adenosyl-L-homocysteine hydrolase	Plays a central role in methylation-based processes by maintaining the intracellular balance between $_S$-adenosylhomocysteine (SAH) and $_S$-adenosylmethionine [78].	[21, 82, 84]
	ndk	Rv2445c	Cytosol and membrane fractions	Probable nucleoside diphosphate kinase NdkA (NDK) (NDP kinase)	Major role in the synthesis of nucleoside triphosphates other than ATP. Secreted Ndk of *M. tuberculosis* acts as a cytotoxic factor for macrophages, which may help in dissemination of the bacilli and evasion of the immune system [15].	[43, 50, 57, 82]
Regulatory proteins	*mce2*	Rv0586	Cytosol and membrane fractions	Probable transcriptional regulatory protein Mce2R (GntR-family)	Transcriptional regulator of *mce2* operon [34]	[57, 58]
	phoP	Rv0757	Membrane and cell wall fractions	Two-component system response transcriptional positive regulator	Positive regulator of the two-component system PhoPR essential for *M. tuberculosis* virulence and potential drug target candidate [87].	[21, 43, 59, 108]
	dosR (devR)	Rv3133c	Cytosol and membrane fraction	Two component transcriptional regulatory protein DevR (DosR)	Controls a regulon of approximately 50 genes: DosR regulon, which is activated by oxygen starvation, nitric oxide, and carbon monoxide [11].	[8, 21, 31, 43, 50, 59, 62, 99, 108]
	senX3	Rv0490	Cytosol	Two-component sensor histidine kinase SenX3	The *senX3-regX3* operon is involved in the *M. tuberculosis* phosphate starvation response and is required for bacterial survival during murine infection [79].	[21, 52, 57, 60]
Secretion systems	*secA2*	Rv1821	Membrane and cell wall fractions	Possible pre-protein translocase ATPase	Accessory secretion factor that promotes growth of *M. tuberculosis* in macrophages. A deletion of the *secA2* gene attenuates the virulence of the organism in mice [54].	[21, 43, 82]
	esxA	Rv3875	Culture filtrate	6 kDa early secretory antigenic target EsxA (ESAT-6)	Exported protein cotranscribed with Rv3874 by the ESX-1 system and is an important virulence factor. Is known to modulate host immune responses and affects antigen presentation function of macrophages [98].	[50, 57, 82, 97]
	esxB	Rv3874	Culture filtrate	10 kDa culture filtrate antigen EsxB (LHP) (CFP10)	Exported protein cotranscribed with Rv3875 by the ESX-1 system and is an important virulence factor [1].	[50, 57 82, 97]
	espA	Rv3616c	Culture filtrate and membrane fraction	ESX-1 secretion-associated protein A, EspA	The secretion of EspA is co-dependent on the presence of the ESAT-6–CFP-10 complex. However, there is no formal evidence that these proteins form a larger complex [1].	[21, 43, 108]
	espD	Rv3614	Membrane fraction	ESX-1 secretion-associated protein EspD	The secretion of EspD is independent on the presence of the ESX-1 system. However, depletion of EspD results in loss of EsxA secretion [12].	[21, 43, 57]
	espC	Rv3615	Membrane fraction	ESX-1 secretion-associated protein EspC	Probably forms an operon with Rv3616c and Rv3614. It is required for the secretion of EsxA and EsxB [1].	[21, 59]

Table 1. Continued.

Category	Gene name	Rv number	Cellular localization	Description	Function	Identified by [a]
Sigma factors	*sigA*	Rv2703	Membrane and cell wall fractions	RNA polymerase sigma factor Sig A (sigma-A)	Initiation factor that promotes attachment of the RNA polymerase to specific initiation sites and then is released. Indispensable for *M. tuberculosis* growth [88].	[43, 59, 108]
	sigG	Rv0182c	Membrane and cell wall fractions	Alternative RNA polymerase sigma factor SigG (RNA polymerase ECF-type sigma factor)	Is one of the most highly induced genes in *M. tuberculosis* during macrophage infection and is required for survival of the bacterium [88].	[21, 52, 60]
	sigH	Rv3223c	Cytosol, membrane, and cell wall fractions	Alternative RNA polymerase sigma-E factor (sigma-24) SigH (RPOE)	Central regulator of oxidative and heat stress responses, it is believed to control protein processing in the extracytoplasmic compartment [88].	[21, 59, 84]
Chaperone proteins	*dnaK*	Rv0350	Cytosol, membrane, and cell wall fractions	Probable chaperone protein DnaK (heat shock protein 70)	DnaK is required for solubility of large multimodular lipid synthases, including the essential lipid synthase FASI; is essential for cell growth and required for native protein folding in *M. smegmatis* [30, 109].	[18, 57, 59, 63, 84, 96]
	hspX	Rv2031c	Cytosol, membrane, and cell wall fractions	Heat shock protein HspX (alpha-crystallin homolog) (14 kDa antigen)	It is a potent ATP-independent chaperone, is highly expressed in latency, and is regulated by the two-component regulatory system DosR [92].	[8, 31, 60, 83, 94, 108]
Fibronectin-binding proteins	*glcB*	Rv1837c	Cytosol and cell wall fractions	Malate synthase G	Involved in the glyoxylate pathway needed for the establishment or maintenance of a persistent infection [95].	[21, 52, 57, 59, 65]
	antigen 85 complex	Rv3804c, Rv1886c, Rv0129c	Culture filtrate and membrane fraction	FbpA, FbpB, FbpC. Secreted antigen 85 (antigen 85 complex) (mycolyl transferase 85) (fibronectin-binding proteins)	Possesses a mycolyltransferase activity required for the biogenesis of trehalose dimycolate (cord factor), a dominant structure necessary for maintaining cell wall integrity. They are one of the major antigens in the immune response to *M. tuberculosis* infection and one of the most abundant proteins in culture filtrate; also, they are fibronectin-binding proteins [51].	[9, 18, 50, 57, 63, 82, 96, 112]
	mpt51	Rv3803c	Culture filtrate	Secreted MPT51/MPB51 antigen protein FbpD (antigen 85 complex D) (mycolyl transferase 85D) (fibronectin-binding protein D)	It might have a role in host tissue attachment; ligands may include the serum protein fibronectin and small sugars [105].	[9, 50, 57, 67, 82, 96]
	apa	Rv1860	Culture filtrate	Alanine- and proline-rich secreted protein Apa (fibronectin attachment protein) (45/47 kDa antigen)	Could mediate bacterial attachment to host cells through its interaction with fibronectin [109].	[41, 57, 58, 82]
	wag22	Rv1759c	Culture filtrate	PE-PGRS family protein Wag22	Could mediate bacterial attachment to host cells through its interaction with fibronectin [28].	[52, 57, 109]

Table 1. Continued.

Category	Gene name	Rv number	Cellular localization	Description	Function	Identified by [a]
Plasminogen-binding proteins	*metK*	Rv1392	Cytosol, membrane, and cell wall fractions	Probable $_S$-adenosylmethionine synthetase (methionine adenosyltransferase)	Catalyzes the formation of $_S$-adenosylmethionine from methionine and ATP, is involved in polyamine biosynthesis during active growth, and is required for the methylation and cyclopropylation of mycolipids necessary for survival in the chronic phase [5, 109].	[43, 59, 62]
	EF-Tu	Rv0685	Cytosol, membrane, and cell wall fractions	Probable iron-regulated elongation factor TU Tuf	EF-Tu is responsible for the selection and binding of the cognate aminoacyl-tRNA to the acceptor site on the ribosome [89, 109].	[43, 50, 61, 84, 108]
	icl 1	Rv0467	Cytosol and cell wall fractions	Isocitrate lyase Icl (isocitrase)	Involved at the first step in glyoxylate pathway needed for the establishment or maintenance of a persistent infection. Deletion of Icl1 and Icl2 resulted in complete impairment of intracellular replication and rapid elimination from the lungs [24, 109].	[50, 65]
	mpt64	Rv1980c	Culture filtrate and membrane fraction	Immunogenic protein Mpt64 (antigen Mpt64/MPB64	Highly conserved protein with unknown function; is expressed only when mycobacteria cells are actively dividing. Probable plasminogen-binding protein [102, 109].	[9, 21, 50, 59, 63, 67, 84, 96]
	groES	Rv3418c	Culture filtrate and membrane fraction	10 kDa chaperonin GroES (protein CPN10) (BCG-a heat shock protein) (10 kDa antigen)	GroES acts in concert with the 60 kDa chaperonin (CPN60), GroEL, ATP, and a repertoire of other molecular chaperones to catalyze protein folding. It is strongly implicated as an important virulence factor during infection [80]. Probable plasminogen binding protein [109].	[43, 59, 63, 65, 82, 96]

[a]It is possible that some proteins described by other authors are not listed here.

plasma membrane proteins and contaminants remain in the supernatant. By following this method, coupled with 1-DSDS-PAGE and nanoLC electrospray ionization MS/MS, it has been possible to identify 349 proteins, 100 of which were integral membrane proteins with at least one predicted transmembrane α-helix.

Triton X-114 has also been used to remove lipids or carbohydrates from the membrane. These components could interfere with the protein resolution [94]. The Triton X-114 method, in combination with nano-ESI-LC-MS/MS for protein extraction, allowed the identification of 43 predicted lipoproteins [58]. It has been suggested that lipoproteins are involved in host-pathogen interactions [97]. These lipoproteins have also been identified as strong immunoantigens for serodiagnosis of tuberculosis [107]. With this methodology, the 10 most abundant lipoproteins in *M. tuberculosis* membranes have been identified, and only three (Rv0432, Rv3763, and Rv0932c) have shown some biological function. These authors also describe one major lipoprotein of *M. tuberculosis*, Rv3623, which has become a pseudogene in *M. bovis* [59].

Shotgun proteomics, labeled and label free, provide a new dimension to proteomic methods [71]. It helps in identifying differences in protein composition between attenuated and virulent strains. In order to compare the membrane protein expression profiles of *M. tuberculosis* H37Rv and its attenuated counterpart H37Ra, Målen *et al.* [60] used orbitrap mass spectrometry technology in combination with relative protein expression abundance calculations. A total of 1,771 different protein groups were identified, of which 1,300 were common to both strains, 278 were exclusively identified in *M. tuberculosis* H37Rv, and 193 were only observed in the H37Ra strain. Although the majority of the identified proteins had similar relative abundance in both strains, 19 membrane and lipoproteins had higher abundance in H37Rv.

Cell Lysates and Cytoplasmic Proteins

After the whole sequence of the *M. tuberculosis* genome, reference maps of cellular fractions have been reported [82]. A subcellular fractionating protocol, which includes sonication, mechanical disruption, and differential centrifugation, was applied to generate 2-DE maps of the cell wall, cytosol proteins, and culture filtrates. In total, 1,184 spots from the three fractions were observed; the protein profile of the three elements was quite different. Sixty-one proteins were identified, and 31 were novel proteins. A genomics non-predicted protein was found, TB9.4, and revealed the presence of an alternative star codon [84]. Another set of six genomics non-predicted proteins have been identified, showing how genomics and proteomics complement each other [49]. Using a different procedure, subcellular fractionation in combination with membrane enrichment using wheat germ agglutinin (WGA) and LC-MS/MS analysis, 1,051 proteins of *M. tuberculosis* were identified, including 183 transmembrane proteins [4].

Because of the complexity of the proteome, proteome-wide studies are still limited in their ability to quantify and identify all proteins. However, recently, the identification of 3,434 proteins from *M. bovis* BCG has been reported, including 512 transmembrane proteins, which represent about 87% of the proteins expressed in the BCG strain. In this study, three subcellular fractions were separated: cell wall, plasma membrane, and cytoplasm. The proteins extracted for each fraction were resolved by 1-D SDS-PAGE. Then, each lane was cut and digested, and peptides were separated by reversed-phase chromatography and analyzed using a LTQ Orbitrap Velos. Up to now, this is the most comprehensive study of the BCG proteome; it will help in the design of vaccination and immunodiagnostic strategies against TB [113].

Culture Filtrate Proteins

Analysis of secreted proteins in culture filtrates has been difficult owing to the tendency of the mycobacterial culture to undergo autolysis with the subsequent release of cytoplasmic proteins to the culture filtrate [82, 103]. However, it was possible to identify some of the major *M. tuberculosis* secreted proteins with the use of a strain that did not undergo the normal autolytic process; the culture filtrates were therefore enriched with secreted proteins [36]. Secreted proteins have been suggested as host protective antigens responsible for the rapid recognition of the bacilli by host lymphocytes. These proteins are available to the immune system more easily when the vaccine used is alive than when it is dead [46]. The most abundant proteins found up to now in culture filtrates have been antigen 85 complex (Ag85A, Ag85B, Ag85C, and MPT51 or Ag85D), MPT63, MPT64, MPT32, MTC28, ESAT-6, CFP10, and others members of the ESAT-6 family [57].

Recently, 103 novel BCG secreted proteins have been reported. The identification of these proteins was accomplished through the use of one-dimensional gel electrophoresis and high-resolution tandem mass spectrometry. These proteins represented potential predominant antigens in humoral and cellular immune responses [115]. Deenadayalan *et al.* [22] identified two new T cell antigens (AcpM and PpiA) through the fractioning of culture filtrates by preparative isoelectric focusing and the analysis of each fraction by immunological studies.

Comparative Proteomics

Proteins present in *M. tuberculosis* but absent in *M. bovis* BCG are valuable antigens for novel diagnostic, therapeutic, and vaccination strategies. Because of this, comparative proteomic analysis focuses on differentiating the protein composition between virulent and attenuated mycobacterial strains. Jungblut *et al.* [50] compared proteins present in two virulent laboratory *M. tuberculosis* strains (H37Rv and Erdman) with those present in two *M. bovis* BCG strains (Chicago and Copenhagen) using 2-DE and MALDI/MS analysis. As expected, the protein composition was very similar, but there were clear differences in spot intensity, and presence or absence and position of the spots. In comparison with BCG, H37Rv comprised 13 additional spots and lacked eight spots. Nine spots had lower intensity, and one spot had higher intensity. Six spots were unique in H37Rv: L-alanine dehydrogenase (Rv2780), isopropyl malate synthase (Rv3710), nicotinate-nucleotide pyrophosphatase (Rv1596), MPT64 (Rv1980c), and two conserved hypothetical proteins (Rv2449c, Rv0036c). Eight proteins were unique to BCG.

Subsequently, using 2-DE and MS, 56 unique proteins were detected in *M. tuberculosis* and 40 in *M. bovis* BCG. Twelve *M. tuberculosis*-specific proteins were identified as products of genes reported to be missing in BCG [60]. The same group compared culture supernatant proteins from *M. tuberculosis* H37Rv and *M. bovis* BCG, and 27 *M. tuberculosis*-specific proteins and 22 novel differential proteins, such as acetyl-CoA C-acetyltranferase and two putative ESAT-6 like proteins, were described [63].

Proteomics has been used to understand differences in

protection of the different substrains of BCG. This has been done by comparing the membrane protein fractions of *M. tuberculosis* and *M. bovis* BCG. The authors were able to detect changes in LpqH, Icl1, and GlcB proteins between these strains. Besides this, it was showed that levels of ESX-3 in BCG were reduced compared with *M. tuberculosis*; and because the ESX-3 system is essential in *M. tuberculosis* with an apparent function in iron and zinc homeostasis, it raises the possibility that the vaccination efficacy of BCG could be improved by increasing the levels of the ESX-3 system [44].

Proteomic analysis of *M. tuberculosis* strains with differences in virulence or drug resistance may provide clues in relation to pathogenicity and help in the identification of virulence factors. Using MS and label-free quantitative proteomic approaches to estimate differences in protein abundance in two clinical Beijing strains, it was found that 48 proteins were over-represented in the hypovirulent isolates, and 53 were over-represented in the hypervirulent. These isolates displayed differences in their level of virulence as defined by their epidemiological and population characteristics as well as virulence in a mouse model of infection. Molecules of cell wall organization and DNA transcription regulatory proteins may have a critical influence in defining the level of virulence [20].

In an effort to determine protein differences between isoniazid resistant and susceptible *M. tuberculosis* strains, Jiang *et al*. [47] compared the proteins extracted from nine isoniazid monoresistant and seven isoniazid susceptible *M. tuberculosis* strains. Five proteins that were up-regulated in isoniazid-resistant strains were characterized by MS: Rv1446c, Rv3028c, Rv0491, Rv2145c, and Rv2971. Most of these differentially expressed proteins are membrane proteins. The gene Rv0491 encodes the protein RegX3, which belong to SenX3-RegX3 of the two-component regulatory system. These systems enable organisms to respond to changing environmental conditions and are involved in *M. tuberculosis* virulence [47, 60].

Latent-Associated Proteins

M. tuberculosis is one of the most successful pathogens of humans. This success is linked to its ability to persist within the human body for long periods of time without causing any symptoms of disease, in a state of latency. The actual state of the bacterium in latency is not well understood; however, different models *in vitro* have been developed in order to mimic the *in vivo* conditions during latent infection. Conditions such as nutrient restriction, low pH, and reduced oxygen tensions have been used. Identification of proteins expressed during latency is important to study the mechanisms of this state. These proteins could represent important targets for vaccine and drug development [11].

The Wayne model of dormancy was used to analyze BCG proteins induced in hypoxic stationary phase cultures. 2-DE analysis showed an up-regulation of the HspX protein, as well as the DosR regulator (Rv3133c) [8]. Metabolic labeling of BCG showed that at least 45 proteins were differentially expressed under standing and shaking culture conditions; the greatest induction was observed with Rv2623, which was expressed under standing conditions but not in shaking cultures [31]. It is interesting that Rv2623 has the ability to regulate growth *in vitro* and *in vivo*, and that it is necessary for the establishment of a persistent infection [23]. Comparative analysis of protein content between aerobic and anaerobic cultures of *M. tuberculosis* showed 13 unique proteins and 37 proteins that were more abundant during anaerobic conditions, some of them related to mycolic acid synthesis and oxidative stress [99]. Two additional proteins (Fba (Rv0363c) and Ald (Rv2780)) were found in large amounts in culture filtrates of *M. tuberculosis* grown under oxygen limitation. These proteins are implicated in glycolysis and cell wall synthesis [83]. Cho *et al.* [13] compared the protein profile of the NRP-1 and NRP-2 stages of metabolic adaptation induced in the Wayne model of hypoxia, applying ICAT technology. They were able to identify 586 proteins in the microaerobic stage NRP-1, and 628 proteins in the anaerobic stage NRP-2. The analysis showed that protein expression decreased in NRP-1 relative to NRP-2, suggesting a temporal down-regulation of the translation machinery necessary for adaptation to low oxygen concentrations.

Through label-free LC MS/MS analysis and applying a nutrient starvation model, 1,176 proteins were identified in culture filtrates of *M. tuberculosis.* The levels of 230 proteins increased in nutrient-starved culture, and the levels of 208 proteins decreased. In this study, proteins of the toxin-antitoxin systems were present in large quantities in nutrient-starved cultures, supporting the hypothesis of its role as metabolic switches [2]. Finally, a comprehensive proteomic analysis of *M. tuberculosis* over the mid-log, early stationary, and late stationary phases was performed. Ten proteins were differentially expressed in the late stationary phase compared with the other two phases, which involve proteins belonging to metabolic pathways and cell wall or lipid biosynthesis [3].

Intracellular Proteins of *Mycobacterium*

M. tuberculosis is an intracellular pathogen that has developed mechanisms to resist the hostile environment inside the macrophage. This resistance involves genes that are potential virulence factors, as well as important proteins for interaction with the host cell. More than that, intracellular mycobacterial proteins can be processed and presented by infected macrophages, so they can represent important protective immune factors; nevertheless, there are only a few studies of mycobacterial proteins expressed *in vivo*, in spite of the valuable information they could provide about natural infection.

The studies of intracellular proteins of mycobacteria are limited because of the difficulties for recovering enough amounts of mycobacterial proteins free from contaminating host cells. Using metabolic labeling of *M. tuberculosis* and infection of THP-1 cells, Lee and Horwitz [55] identified 44 proteins expressed differentially, where 16 were up-regulated and 28 down-regulated. Of the 16 up-regulated proteins, six were absent during extracellular growth under normal and stress conditions. In a different study, six abundant proteins showed increased expression inside the macrophage: 16 kDa α-crystallin (HspX), GroEL1, GroEL2, Rv2623, InhA, and elongation factor Tu (Tuf). In this study, it was also shown that most of the intracellular modulation of protein expression occurred within the first 12−24 h following phagocytosis, reflecting the dynamic intracellular environment during the interaction between the cell and the bacilli [66]. Using subcellular fractionation of infected macrophages to isolate phagosomal *M. tuberculosis*, in combination with high-resolution 2-DE and MALDI-MS, 11 exclusively intraphagosomal mycobacterial proteins were detected. Some were involved in metabolism and cell envelope synthesis [64].

In order to understand whether the mechanisms adopted by resistant and sensitive mycobacteria to survive and grow inside the macrophage are similar or different, Singhal *et al*. [93] analyzed the protein profile of *M. tuberculosis* MDR and sensitive clinical isolates infecting THP-1 cells. Mass spectrometry and bioinformatic characterization showed that the majority of commonly expressed proteins belonged to the cellular metabolism and respiration category. More than that, some common proteins were found to be overexpressed, so it is possible that some common mechanism is adopted by sensitive and resistant strains for their survival inside the macrophage. Understanding this mechanism could be useful in drug targeting [93].

Recently, the first proteomic characterization of *M. tuberculosis* during infection *in vivo* in a guinea pig model by aerosol infection was reported, and over 500 proteins present at 30 and 90 days post infection were described. Two functional groups represent about half of the total proteins identified: category 3 (cell wall and cell process) and category 7 (intermediary metabolism and respiration). These proteins displayed high heterogeneity, indicating important biological processes necessary in different stages of infection. Many proteins identified in this study may provide the basis for rational drug design [52].

PPD Protein Composition

Tuberculin skin testing (TST) is the gold standard for determining whether an individual is infected with TB. This test involves the intradermal injection of a purified protein derivative (PPD), which is a poorly defined mix of proteins, and little is known regarding which of these components are responsible for the DTH response. A complete knowledge of the molecular composition of the PPD will allow the selection of proteins specific for a more refined test to human and bovine TB. However, little has been done to identify the components of the PPD, because of the degradation, partial denaturation, and aggregation of many of the protein components [110].

Borsuk *et al*. [9] characterized the proteins present in bovine and avium PPD from Brazil and UK. A total of 171 different proteins were identified, most of them cytoplasmic proteins related to intermediary metabolism and respiration. Most secreted proteins were present in the PPD preparation: Ag85 complex, MPT32, MPT64, MPT83, MPT53, and MPT70. Some differences between PPD preparations from Brazil and UK were observed, probably due to differences in preparation methods and these partially could explain the differences in biological potency of PPD products from different sources [110]. Twenty-one proteins were identified in both bovine PPD preparations, but not in *M. avium* preparations, where 10 of these were not present in *M. avium*; therefore, these proteins are candidates to differentiate exposure of the animal to environmental mycobacteria from bovine tuberculosis [9].

A proteomic analysis of the FDA standard PPD-S2 showed that it is composed of at least 240 proteins, and many of the known *M. tuberculosis* T cell antigens dominated the PPD composition (GroES, GroEL2, HspX, and DnaK). Additional comparative proteomic and histological analysis between PPD-S2, RT23, and PPD-KIT demonstrated that differences in the relative abundance of several proteins, including members of the Esx protein family, may contribute to the

increased inflammatory responses observed with RT23 and PPD-KIT reagents, supporting the theory that variability in PPD reagents may explain the differences in DTH responses reported among populations [14]. Protein analysis of PPD-CT68 (Tubersol) showed that 142 proteins were shared between PPD-CT68 and PPD-S2 preparations, whereas 123 and 89 were exclusively identified from PPD-CT68 and PPD-S2, respectively. Eighteen proteins were common in PPDs from *M. tuberculosis*, *M. bovis,* and *M. avium* [75].

Despite the identification of many antigens, it is still challenging to replace classical PPD preparations. Currently, ESAT-6, CFP-10, Rv3615, DnaK, and GroEL2 are under evaluation as next-generation PPD candidates in the diagnosis of human [6, 111] and bovine TB [32, 91, 101].

M. tuberculosis is a very successful pathogen that has a very complex interaction with the host cell, where it can persist for long periods without causing any symptoms of disease. WHO estimates that almost one-third of the world population is latently infected with *M. tuberculosis* and this latency is a constant source of disease reactivation. Five to ten percent of the infected individuals develop active TB over their lifetime, and defects in cell-mediated immunity, HIV co-infection, malnutrition, administration of chemotherapy or antitumor necrosis factor therapy, and diabetes predispose latently infected people to develop TB [16]. Therefore, it is quite important to develop new diagnostic reagents, new vaccines, and new targets for TB drugs. Proteomics can have a significant impact on our current understanding of *M. tuberculosis*, especially about its pathogenicity, virulence, and interactions with host cells.

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