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Proteome characterization of the culture supernatant of *Mycobacterium bovis* in different growth stages

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ABSTRACT

This study aimed to identify proteins secreted by *Mycobacterium bovis* into culture medium at different stages of bacterial growth. A field strain of *M. bovis* was grown in Middlebrook 7H9 media and culture supernatant was collected at three-time points representing three different phases of growth (early exponential, late exponential, and stationary phases). Supernatants were double filtered, digested by trypsin and analyzed by LC-MS/MS. The study found 15, 21, and 16 proteins in early, mid and late growth phases, respectively. In total, 22 proteins were identified, 18 of which were reported or predicted to have a cell wall or extracellular localization. To our knowledge, this is the first study to identify proteins secreted into the culture medium by a field strain of *M. bovis* in three different stages of growth. The dataset generated here provides candidate proteins with the potential for the development of serological diagnostic reagents or vaccine for bovine tuberculosis. Data are available via ProteomeXchange with identifier PXD017817.

1. Introduction

Bovine tuberculosis (BTB) is a chronic zoonotic infectious disease that affects all mammals and contributes to significant global economic losses, which are estimated to be about US$ 3 billion annually [1–3]. Attention has been given to the identification of immunogenic proteins of *M. bovis* origin that can be used for diagnostic reagent development. A well-established approach is the analysis of the Purified Protein Derivative, a standardized preparation of the culture filtrate of the *M. bovis* AN5 strain, (PPDB) [3]. PPDB is used to assess the cell-mediated immune response to infection with *M. bovis*, being the reagent used in both the Tuberculin skin test (TST) and the Gamma-interferon test, the major diagnostic tests of BTB [4,5]. Most previous studies focused on the analysis of the PPDB components to define possible T-cell stimulants that contribute to the development of the delayed hypersensitivity reaction in the TST or produce a reaction in the Gamma-interferon assay [6–8]. Borsuk et al. [9] reported a majority of cytoplasmic proteins (77.9%) in the analysis of PPDB which may be related to the heat killing procedure used in PPDB preparation. This study aims to provide new insight into the identities of proteins secreted into culture medium by *M. bovis* in three different growth phases. Here, the heat-killing of bacterial culture was not implemented in order to minimize the release of cytoplasmic proteins. The proteins identified here may be involved in the humoral or the cellular immune response to *M. bovis* infection and may prove to be candidates for vaccine or novel diagnostic antigen discovery.

2. Materials and methods

2.1. Mycobacterium bovis growth and culture supernatant harvest

One ml of concentrated, previously frozen *M. bovis* isolate 2011/0690 was inoculated into 20 ml Middlebrook 7H9 with albumin-dextrose-catalase (ADC) enrichment and incubated at 37 °C. This is a virulent *M. bovis* strain that was isolated from BTB outbreak that affected beef cattle in British Columbia, Canada in 2011. The genome sequence of this isolate, unpublished data, is highly similar to the published *M. bovis* strain 2011/0565, another isolate from the same BTB outbreak with close similarity to the reference *M. bovis* strain, AF2122/97 [10]. After 10 days, 1 ml of the culture was inoculated into 10 ml vials of 7H9 liquid medium- ADC enriched. The vials were incubated at 37 °C with continuous shaking at 300 rpm to avoid bacterial clumping. The OD at 600 nm in such conditions was measured for the bacterial cultures and considered reliable as previously described [11–13]. The addition of Tween 80 was dismissed because it was associated with a reduction in

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Abbreviations

Ag	Antigen
TB	tuberculosis
BTB	bovine tuberculosis
PPD	Purified Protein Derivative
BCG	Bacille Calmette Guerin

the virulence of *M. tuberculosis* and possible detrimental effects on the mycobacterial cell wall structure [12]. At least three vials were used to represent three time points: 1) early exponential phase (growth for 10 days); 2) late exponential phase (growth for 21 days); and 3) stationary phase (growth for 42 days). To minimize the release of cytoplasmic bacterial proteins into the growth media, the bacterial culture was not heat-treated. Instead, the cultures were sterile filtered twice to obtain the culture supernatant samples, free of viable mycobacteria. The filtration was performed using Vivaspin centrifugal concentrators (Sartorius) with 0.2 μm filter that were centrifuged at 1000 g for 5 min, and the supernatant collected from each vial was then passed through a sterile, 0.22 μm syringe filter to ensure sterility and absence of any viable bacteria. Each growth stage was represented by at least three biological repeats. Optical density measurements ranged from 0.45 to 0.55 for the early exponential phase., from 0.71 to 0.73 for the late exponential phase. and 0.8–0.86 for the stationary phase. It was noticed that the doubling time seen here was long compared to another strain grown in the laboratory, Fig. S1. The doubling time for *M. tuberculosis* H37Rv strain was reported as 24–96 h [14,15], however, one report showed the doubling time of *M. tuberculosis* H37Rv to be 5 days [11]. Another report showed that clinical isolates of *M. tuberculosis* were slower in growth when compared to the lab trained *M. tuberculosis* H37Rv strain [16] and this agrees with the slow growth of the field isolate seen here. Sterility was further assessed by culturing 500 μl of each supernatant sample into 7H9 medium- ADC enriched then Loewenstein-Jensen medium at 37 °C for 35 days. Sterility was confirmed when no bacterial growth was observed with the supernatant samples, indicating that the materials to be used for proteomic analysis are free of viable mycobacteria.

2.2. Sample preparation for the proteomic analysis of M. bovis proteins secreted into the culture medium

Bovine albumin depletion from the culture supernatant. Bovine albumin contained in the culture supernatant samples was depleted prior to proteomic analysis using a Pierce Albumin Depletion Kit (Thermo Scientific, USA) following the manufacturer's instructions.

Protein precipitation. The culture supernatant proteins were precipitated using 10% TCA (v/v), allowed to precipitate overnight in a −20 °C freezer, then spun for 30 min at 10,000×*g* at 4 °C. The supernatant was removed, and the pellet was washed twice using 50% ether in 50% acetone (ice cold). The sample was centrifuged again for 5 min at 10,000×*g* to remove the supernatant, and the residual supernatant was left in the air to evaporate.

Reduction and Alkylation. The dry protein pellet was solubilized and denatured using 7 M urea in 50 mM ammonium bicarbonate and ProteaseMAX™ Surfactant (Promega, USA) to help protein solubilization (~0.05% w/v). Dithiothreitol (DTT) was added to reduce disulfide bridges at a final concentration of 10 mM. Iodoacetamide (IAA) was added to alkylate free cysteines at a final concentration of 25 mM. Protein is quantitated using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). The protein was diluted 5 times in 50 mM ammonium bicarbonate to bring the urea concentration down to 1 M.

Trypsin Digestion and Peptide Purification. Trypsin Gold, Mass Spectrometry Grade, (Promega, USA) was used at a 1:20 ratio of trypsin mass to protein mass, for 100 μg of protein. ProteaseMAX™ Surfactant (Promega, USA) is added to a final concentration of 0.05%. Digestion was done overnight at 37 °C and quenched by adding TCA to a final concentration of 10%. This was followed by centrifugation at 16,000 g for 10 min. Peptides were purified from the soluble fraction using Pierce C18 columns (Thermo Scientific, USA) as per the manufacturer's instruction. Peptides were eluted from C18 columns using 70% (v/v) acetonitrile which is later removed with a Spin Vac (ThermoFisher Scientific, USA). LC-MS/MS analysis and data processing are described in the Supporting Information. An automated decoy search was performed to obtain false-positive rates of approximately 1%. Proteins were searched against *M. bovis* Swiss-Prot database. Protein quantitation was done using a label-free quantitation. The gene loci were reported from the *M. bovis* reference strain, *M. bovis* AF2122/97 [17].

3. Results and discussion

Twenty-two proteins were detected from different stages of bacterial growth (Table 1). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [18] partner repository with the dataset identifier PXD017817. Proteins reported here were detected in at least two biological repeats. The experiments were done primarily on albumin-undepleted samples then repeated using albumin depleted media to improve the mass spectrometric detection of *M. bovis* proteins. Albumin depletion caused an increase in the number of detected proteins from 8 to 15 proteins at 10 days of bacterial growth, 15 to 21 proteins at 21 days of growth, and 15 to 16 proteins at 42 days of growth. Out of the total 22 proteins detected, 18 (81.8%) were reported or predicted to be secreted, cell wall or extracellular. Eight out of the 18 had signal peptides (Table 1). The total number of proteins detected in the late exponential or the stationary phase is lower than what is reported in other studies [7–9,19,20]. This might be due to the fact that this study used a field strain, whereas the previous studies used laboratory-adapted strains, *M. bovis* AN5 and *M. tuberculosis* H37Rv. Another factor may be related to different media used to culture bacteria. The previous studies use Sauton or Reid media (albumin or protein poor) whereas this study used albumin-enriched medium.

Twelve proteins were consistently detected in the three stages of bacterial growth. Of these, the most abundant secreted proteins are CFP-10 (Mb3904), ESAT-6 (Mb3905), MPB70 (Mb2900) and the MPB83 (Mb2898). Interestingly, MPB70 and MPB83 identified in the PPDB analysis are currently used in the BTB serological tests as in the USDA-approved IDEXX TB ELISA [21,22]. CFP-10 and ESAT-6 have been employed in the Gamma-interferon assay to increase assay specificity and to differentiate BCG-vaccinated animals from unvaccinated ones [23]. Some countries such as the United Kingdom and New Zealand are using these antigens to confirm or negate the results of TST (serial TB testing) [22,23]. The abundant proteins shown in this study were similar to those reported by Roperto et al. [7] and Rennie et al. [24]. The present study also identified proteins Ag85A and Ag85B, similar to the findings of Rennie et al. [24], but not Ag85C. This difference could be because the strain used in this study may secrete Ag85C at a level below detection limits of our experimental setup. Ag85 proteins are known to have variable abundances among different *M. tuberculosis* strains [25]. The Ag85A, Ag85B and Ag85C play a role in the affinity of mycobacteria for fibronectin and have been previously considered as candidates for vaccine development [26].

Furthermore, three heat shock proteins from *M. bovis*, GroL2 (Mb0448), HspX (Mb2057c), and DnaK (Mb0358), were identified in this study. GroL2 was continuously detected in all growth stages from the early until the stationary phase. HspX and DnaK were detected in the latter two stages. The homologous counterparts of these three proteins (GroEL2, HspX, and DnaK) were reported by Cho et al. [19] after the analysis of the PPD from *M. tuberculosis*. The latter study showed that these proteins dominated the composition of PPD. These proteins are

Table 1
A summary of proteins detected in the culture supernatant in different growth phases of *M. bovis* isolate 2011/0690.

Mb#[a]	Detection stage[b]			[c]Localization	[d]Signal peptide	Size (kDa)	Protein name
	Early Exp	Late Exp	Stationary				
Mb3904	+	+	+	S	N	10.8	CFP-10
Mb3905	+	+	+	S	N	9.9	Early secretory antigenic target (ESAT-6)
Mb2900	+	+	+	S	Y	19.1	MPB70
Mb0448	+	+	+	C/S	N	56.7	60 kDa chaperonin 2- GroL2
Mb2268	+	+	+	C	N	12.5	Meromycolate extension acyl carrier protein
Mb2898	+	+	+	S/CW	Y	22.1	Cell surface glycolipoprotein MPB83
Mb1918c	+	+	+	S	Y	34.5	Antigen 85 complex B (Ag85B)
Mb2397c	+	+	+	S	Y	16.6	Low molecular weight antigen MTB12
Mb2002c	+	+	–	S	Y	24.8	Antigen MPB64
Mb0192A	+	+	+	U	N	5.7	Metallothionein OS
Mb1858	+	+	+	CW/EC	N	17.3	Uncharacterized protein Mb1858
Mb1891	+	+	+	S	Y	32.7	Fibronectin attachment protein (FAP-B)
Mb2970c	+	+	+	CM/CW/S	Y	24.1	Lipoprotein LppX
Mb3834c	+	+	–	S	Y	35.7	Antigen 85 complex A
Mb0358	–	+	+	C/ECV	N	66.5	Heat shock protein 70 - DnaK
Mb1967	–	+	+	C	N	16.9	Thiol peroxidase (TPX)
Mb1821	+	–	–	S/EC/PM	N	9.9	ESAT-6-like protein EsxN
Mb1662	–	+	–	CW/PM	N	15.3	Iron-regulated universal stress protein family protein-TB15.3
Mb0055	–	+	–	EC/PM	N	17.7	Single-stranded DNA-binding protein
Mb3672c	–	+	–	C/CW	N	7.4	Probable cold shock protein A cspA
Mb2057c	–	+	+	CW/EC	N	16.2	14 kDa antigen- HspX-HSP 16.3
Mb3945	–	+	+	C	N	12.5	Thioredoxin/MPT46

[a] Mb# refers to protein name in *M. bovis* AF2122/97.
[b] Exp – Exponential; "+" refers to protein detected in this stage; "-" refers to protein not detected at that stage.
[c] C– Cytoplasmic; CW– Cell wall; EC– Extracellular; ECV– Extracellular vesicle; PM– Plasma membrane; S– Secreted; U– Unknown.
[d] Y– Signal peptide present; N– Signal peptide absent.

cytoplasmic and possible T cell antigens, but they were also reported to be in the bacterial extracellular space or on the cell surface [20,27,28].

Additional identified proteins that may have extracellular localization are Mb2970c (LppX) that functions in lipid transport and Apa (Mb1891), a protein with a role in extracellular matrix binding [29,30]. Two other extracellular proteins that may play a role in the development of immune responses to mycobacterial infection, MTB12 (Mb2397c) and MPB64 (Mb2002c), were also identified [31]. MPB64 is a possible plasminogen-binding protein that may have a role in the bacterial virulence [32]. Mb1858 is another possible extracellular protein detected in this study and is a presumed regulator of glutamate metabolism [28,33].

A number of proteins were only detected in the early phases of growth but not in the stationary phase. This observation is possibly due to protein degradation. One secreted protein, ESAT-6-like protein EsxN (Mb1821), of unknown function, was found only in the early exponential phase. Two proteins with possible extracellular localization, Mb1662, a stress protein, and Mb0055, a single-strand DNA binding protein, were only detected in the late exponential phase; both have been reported to play a role in antibiotic response [34].

This study detected two proteins that have not been previously identified by analyzing PPDB preparations [7–9]. The first is Mb0192A, a metallothionein detected in three phases of growth. The second is Mb3672c, a probable cold shock protein A, of possible cell wall localization that was detected only in the late exponetial phase of growth. Mb3672c was reported to be of high structural fragility [35,36].

This study is the first to characterize a field *M. bovis* strain for the proteome of its culture supernatant in three growth stages, aiming to provide insight into possible extracellular proteins secreted by the bacterium, which may have helped identify protein candidates useful for diagnostic reagent or vaccine development.

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The authors have declared no conflict of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101154.

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