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Secretome differences between the taxonomically related but clinically differing mycobacterial species *Mycobacterium abscessus* and *M. chelonae*

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Abstract

Rapidly growing non-tuberculous mycobacteria (NTM) are significant human pathogens which show high inter-species differences in clinical characteristics (virulence, host immune response) during infection even within a given NTM complex. Understanding the differences between the secreted proteomes of the member species for an NTM complex may reveal the basis of their differential virulence and host pathogenesis potential including host immune reactions. In this study, major secreted proteins of the two taxonomically close but clinically differing member species *M. abscessus* and *M. chelonae* of the *M. chelonae-M. abscessus*(MCA) complex were compared using an approach based on 2-dimensional gel electrophoresis (2-DE) and MALDI-TOF analyses. The two secretomes showed dramatic differences. Of the 73 major secreted proteins identified, majority were expressed in a species-specific manner, including 37 in *M. chelonae* and 32 in *M. abscessus*. Interestingly, 9 of these differentially expressed proteins were orphan proteins showing homology to either hypothetical proteins or those with no defined function. The other 60 distinctly expressed proteins were homologs of those associated with various bacterial cellular functions and virulence, namely cell wall synthesis or lipid metabolism, metabolic and respiratory pathways, stress response and signal transduction, gene regulation, and immune response. This information on species-specific secreted proteins would help understand the critical virulence factors and host pathogenesis mechanisms in these mycobacterial species and provide the basis for developing better therapeutic strategies. These proteins may also serve as potential targets for species-specific diagnosis as an additional outcome. To our knowledge, this is the first attempt to characterize the secretome of *M. chelonae* (for which the genome sequence is not yet available) and the secretome differences between *M. abscessus* and *M. chelonae*.

Keywords: Secretome; Secreted proteins; 2-DE; *Mycobacterium; M. chelonae; M. abscessus* .

Abbreviations:

2DE, Two-dimensional gel electrophoresis; **RGM**, rapidly growing mycobacteria; **NTM**, non-tuberculous mycobacteria; **MCA**, M. chelonae-M. abscessus; **SGM**, slow growing mycobacteria; **MB7H10**, Middle brook 7H10; **ATCC**, American Type Culture Collection.

1. Introduction

Non-tuberculous mycobacteria (NTM) are ubiquitously distributed in hospital and other environments such as drinking water, soil, biofilms, aerosols, swimming pools, and metal working fluids [1]. There have been increasing reports of NTM infections in both immunocompetent and immunocompromised individuals particularly HIV patients [2, 3]. While majority of the past studies on virulence factors and mechanisms in NTM have been focused on slowly growing

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mycobacteria (SGM) group particularly *Mycobacterium avium* complex [3], little is known on the rapidly growing mycobacteria (RGM) species. Among the RGM, the *M. chelonae-M. abscessus* (MCA) complex is particularly significant considering its frequency of occurrence in clinical conditions and extraordinary drug resistance. *M. abscessus* and *M. chelonae* are the original members of the MCA complex that are most frequently detected in clinical conditions associated with this complex [3, 4]. Recently, *M. immunogenum* and two former subspecies of *M. abscessus* viz. *M. bolletii*, and *M. massiliense* have been added as new member species under this complex [5, 6, 7]. The MCA species are genetically closely related and thus require development of specialized diagnostic strategies to differentiate their clinical and environmental isolates, as reported in our recent efforts [8, 9].

Species of the MCA complex are opportunistic pathogens that have been reported to cause several pseudo-outbreaks [10] and chronic infections in lung or other tissues (skin, soft tissues, liver, joints, and female genital tract) of the human body [2]. Many of the infections occur inside the body after normal surgery procedures[11-17]]. Pulmonary diseases caused by the MCA species [3], manifest as infection and/or immunological pathologies. The infection pathologies are characterized by tuberculosis (TB)-like symptoms [2]. On the other hand, species of this complex have been implicated as etiological agents of the immune-mediated lung disease hypersensitivity pneumonitis (HP) in machinists exposed to contaminated machining fluids [18]. In this context, our laboratory has reported the occurrence of multiple genotypes of MCA species *M. chelonae*, *M. immunogenum* and *M. abscessus*, from these fluids [19, 20].

Despite being taxonomically closely-related, member species of the MCA complex vary in their clinical presentation both in terms of disease potential/severity and drug resistance. *M. abscessus*, in particular, is a newly emerged pulmonary pathogen increasingly being isolated from cystic fibrosis and other respiratory patient populations [21]. It is now the most frequently encountered species in RGM case reports and is the causative pathogen in an estimated 80% cases of the RGM pulmonary disease [22]. Collectively, these clinical epidemiological studies imply a relatively greater potential of *M. abscessus* in causing MCA pulmonary infections. Independent experimental studies on its pathogenesis potential have shown that *M. abscessus* can replicate in vitro in cultured macrophages and multiply or persist in a mouse model of infection [23]. In contrast, *M. chelonae* showed early clearance both in the infected macrophages [23, 24] and a mouse pulmonary infection model (Unpublished data). Other studies using mouse intravenous infection models demonstrated differential immune control (clearance) of M. abscessus versus M. chelonae infection [25]. Collectively, the above discussed clinical/phenotypic differences between *M. abscessus* and *M. chelonae* are expected to result from the differences in the underlying molecular factors in these species. However, such virulence and host response-determining factors in these species of the MCA complex have not yet been characterized.

Secreted proteins are known to play important role in the survival of tuberculous species inside the host or in the environment. Several secreted proteins with specific role in virulence have been reported from *M. tuberculosis* and related SGM species [26, 27]. However, little information is available on the secretomes of non-tuberculous mycobacteria particularly the RGM species of clinical significance [22]. We recently published the immunoproteome (including secreted antigens) of the MCA species *M. immunogenum* [28]. The aim of the current study was to identify and compare secreted proteins of the two prominent and clinically significant member species *M. abscessus* and *M. chelonae* of this complex. The experimental approach included proteomic profiling of their culture filtrate proteins based on 2-DE and MALDI-TOF analyses, and comparing the two secretomes for shared and unique proteins and their putative functional relevance. Recently, the complete genome sequence of *M. abscessus* has been reported [29] (http://www.ncbi.nlm.nih.gov) whereas the genome sequence of *M. chelonae* is not yet available. This study on secretome analysis in these two closely-related MCA species will complement the post-genomic analysis and help in studying their systems biology for understanding the virulence and host pathogenesis differences. The identified secreted proteins may have importance in the development of novel therapeutics and vaccine candidates.

2. Materials and Methods

2.1 *Strains and culture conditions*

M. abscessus ATCC 23006, a lung isolate from a patient's sputum, and *M. chelonae* ATCC 35752, the type strain originally isolated from tortoise, were obtained from the American Type Culture Collection (ATCC). Both species were maintained on Middle brook 7H10 (MB7H10) agar and cultivated using Sauton's medium. For proteomic studies, liquid cultures of the two species were grown to log phase under identical conditions, using Sauton's broth and incubation by shaking (250 rpm) at 37 ˚C.

2.2 *Preparation of secretory proteins extract*

Bacterial cells from either species were pelleted by centrifugation (3000g) at 4˚C for 30 min and the culture supernatant was clarified by passing through a 0.22 μm membrane filter. The filtrate was concentrated by trichloroacetic acid (TCA) protein precipitation method [28] followed by resuspension of the precipitate in rehydration buffer (9 M urea, 2M thiourea, 4% CHAPS, 65mM DTT) to isolate the secretory protein extract. Protein concentration in the resulting protein extract was determined using Quick Start Bradford protein assay kit (Bio-Rad, Hercules, CA), according to the manufacturer's specifications [30].

2.3 *Isoelectric focusing and two-dimensional gel electrophoresis (2-DE)*

For the isoelectric focusing (IEF) prestep, 100 µg of the protein extract was added to 125 µl of rehydration buffer (9 M urea, 2M thiourea, 4% CHAPS, 65mM DTT, 0.5% IPG buffer and 0.002% bromophenol blue). The IEF separation was achieved using a 7cm IPG strip pH range 4-7 (GE Healthcare, Piscataway, NJ) on the IPGphore II isoelectric focusing system (GE Healthcare, Piscataway, NJ). The strip was rehydrated using 50µA for 12h at 15°C and then focused for 16000 volt hours using an upper current limit of 50 µA. Prior to running the 2D gel, the IPG strip was incubated twice for 10 min each in equilibration buffer (6 M Urea, 30% glycerol, 0.05 M Tris pH 8.8, 2% SDS, 0.002% bromo phenol blue), first in conjunction with 135 mM DTT and then with 135 mM iodoacetamide.

The IEF separated proteins were subjected to 2-DE using 15% SDS-PAGE gel. The protein gels were stained with SYPRO Ruby (Invitrogen, Carlsbad, CA) and the gel images were taken and processed using the 2D imaging software Imagemaster ™ 2D Elitegive version 4.01 (GE Healthcare, Piscataway, NJ). The isolated protein spots were manually picked from the SYPRO Ruby-stained gel with one touch spot picker (The Gel Co., San Francisco, CA) and prepared for mass spectroscopy analysis, as described below.

2.4 *MALDI-TOF peptide mass mapping*

The well separated protein spots on the 2D gel were excised and chopped into small pieces followed by 3 alternate cycles of washing using 500 µl of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate buffer pH 8.0 and dehydration using 100% ACN. The treated gel pieces were dried completely by vacuum centrifugation followed by rehydration at 4˚C for 1h with 25 µl trypsin digestion buffer (100 mM ammonium bicarbonate buffer and 1 mM $CaCl_2$) containing 50 ng trypsin gold (Promega, Madison, WI, USA). Extra trypsin buffer was then added for complete submerging of the gel and the reaction mixture incubated at 37˚C with continuous shaking for 16h. Following centrifugation, the individual gel slices were subjected to protein elution for 10 min using 50µl of 5% trifluoroacetic acid (TFA) in 50% ACN (v/v). This process was repeated and the extracts were pooled. The eluted digested protein sample was then evaporated by vacuum centrifugation and kept at -20˚C until use. Just before analysis, the digested protein samples were dissolved using 5% TFA in 50% ACN.

The MALDI-TOF analysis was performed on a PE Voyager DE_STR biospectrometry work station (Applied Biosystems, Foster city, CA) set in reflector mode in the mass range 500 to 5000 Da. The matrix α-cyano-4-hydroxycinnamic acid (CHCA) was prepared in a saturated solution of 50% ACN-0.1% TFA. For the mass spectral analysis, equal volumes of the matrix solution (1µl) and the sample (1µl) were mixed on the sample plate, and air dried to form crystals. Each peptide mass spectrum was calibrated using external standards from Sigma (Insulin oxide β, ACTH Fragment, Angiotensin I, Bradykinin) and internally calibrated with trypsin autolysis peaks; mono isotopic peaks of trypsin auto digests were 842.508, 1045.504 and 2211.108, 2225.12 m/z.

2.5 Bioinformatic analysis

An automated analysis of the mass peaks was done against the protein database using MASCOT search engine (http://www.matrixscience.com/search_form _select. html). To assign a positive identification, at least three peptides had to match, with a search tolerance of 100 ppm while allowing one miscleavage as used in a previous study [28]; possible fixed modifications ascribed to alkylation of cysteine by carbamidomethylation and oxidation of methionine were taken into consideration. For the probability based peptide mass fingerprinting identification, a minimum of one significance hit ($P<0.05$) was considered as an identity. In some instances, the protein was identifiable despite the low mass score because its top hit was a mycobacterial protein (which allowed it to be differentiated from other proteins). The bioinformatics approach for protein identification is demonstrated in the online Supplementary Information showing tRNA pseudouridine synthase protein identification of *M. chelonae* as an example (supplementary file 1).

2.6 *Mycobacterial database searching*

The MASCOT-identified proteins were characterized using the available mycobacterial databases namely Tuberculist (www.sanger.ac.uk), Proteome 2D-PAGE Database (http://web.mpiib-berlin.mpg.de/cgi-bin/pdbs/2d-page), the recently released whole genome sequence database for *M. abscessus* (EMBL accession numbers: CU458896, chromosome; CU458745, plasmid), and the published information on mycobacterial and other bacterial proteins. Function-based profiling of the identified secreted proteins was done on the basis of available database and literature mining, mainly using the functional annotation database developed by the Sanger Centre of Genomic Research (http://www.sanger.ac.uk/Projects/M_tuberculosis/Gene_list) and the published literature retrieved via PubMed.

3. Results and Discussion

Investigation of secretomes offers a unique tool to investigate the pathogen factors playing a direct role in host-pathogen interaction and clinical manifestation of infection. This study compared the secretome profiles of *M. abscessus* and *M. chelonae* based on 2-DE and MALDI-TOF analysis of the culture filtrates. The comparisons were made in terms of differential secretion of the protein factors known to be of relevance in pathogen virulence and host response including immune reaction in mycobacteria and other bacterial pathogens (see Tables 1 and 2 and Figures 1-3).

3.1 *Optimization of 2-DE separation of the secreted proteins*

For efficient comparative secretome analysis, the 2-DE separation of the culture filtrate proteins from the two mycobacterial species was first optimized by varying the IEF pH range and SDS-PAGE concentrations. We observed a better resolution of the proteins in the acidic pH range 4-7 than in a broader pH range (pH 3-10) and in 15% SDS-PAGE, for both the mycobacterial species. More than 100 proteins were visualized on the SYPRO Ruby-stained gel, run in triplicate, for either species (Figures 1A and 1B). Major protein spots manually excised from the 2D gels and trypsin digested were reliably identified by MALDI-TOF mass spectroscopy and database searching (Tables 1 and 2).

3.2 *Functional grouping of the secreted proteins*

Majority of the identified secreted proteins were found to be the homologs of the proteins that play diverse roles in both pathogenic and nonpathogenic bacteria. These identi-

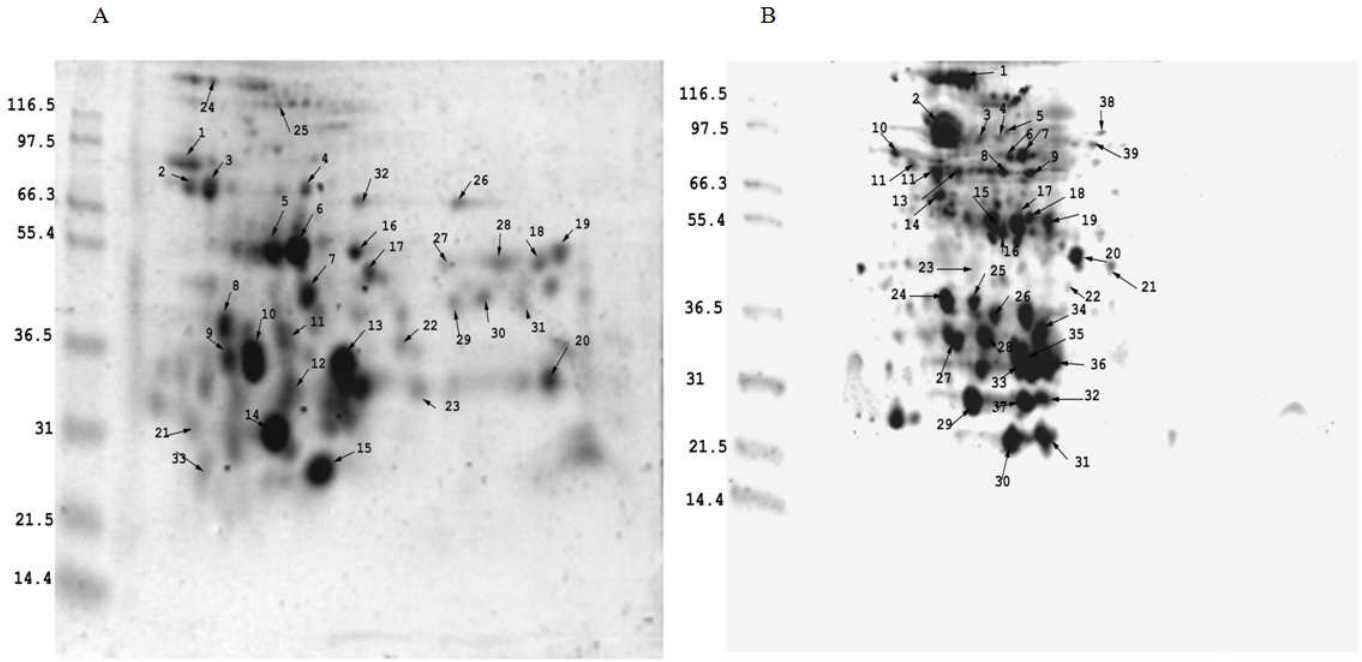

Figure 1. Two-dimensional gel electrophoretic (2-DE) separation of the secretome proteins (A). *M. abscessus* (ATCC 23006). (B). *M. chelonae* (ATCC 35752). Culture filtrate proteins prepared from the actively growing log-phase cells of either species were separated on 2-DE gels (IEF pH gradient 4-7) and stained with SYPRO Ruby as described under Materials and Methods. The approximate positions of molecular weight markers (kDa) are indicated. The spot numbers indicated on the gel correspond to the protein numbers presented in Tables 1 and 2.

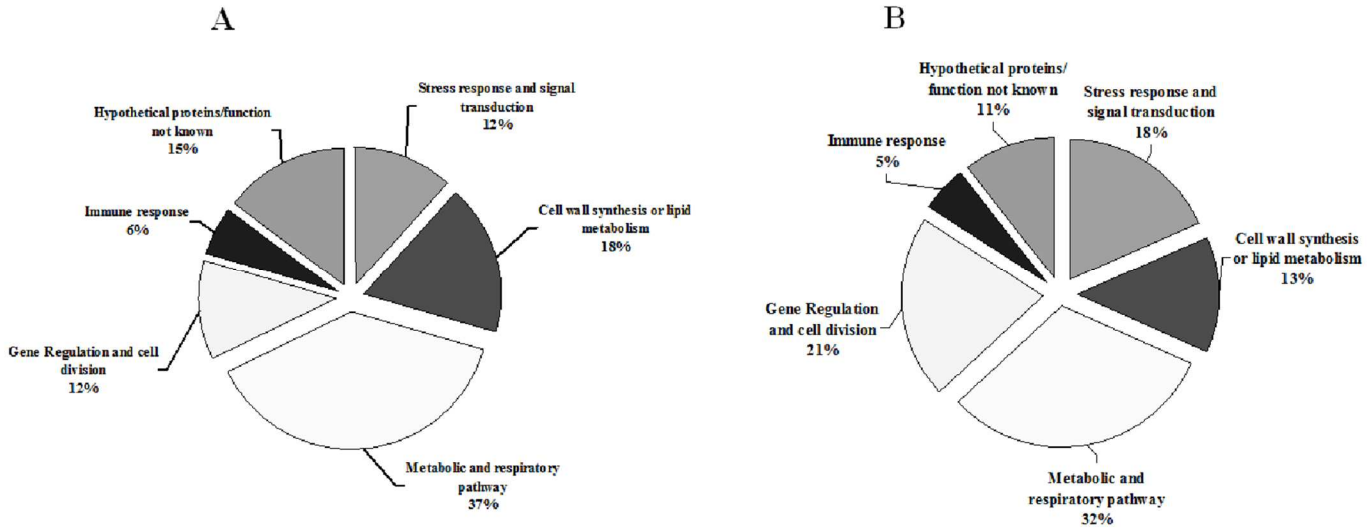

Figure 2. Functional distribution of the identified secreted proteins. (A) *M. abscessus* (ATCC 23006). (B) *M. chelonae* (ATCC 35752).

fied protein homologs in the two species could be categorized in six different functional groups (Figures 2A and 2B), namely (1). Cell wall synthesis or lipid metabolism; (2) Stress response and signal transduction; (3). Metabolic and respiratory pathways; (4). Gene regulation and cell division; (5). Immune response ;(6). Hypothetical proteins of unknown function. Some of the secreted proteins could be assigned to more than one of these functional categories. Relative percent distribution of the individual functional categories in the two secretomes is presented in Figure 2.

3.3. *Comparison of the M. abscessus and M. chelonae secretomes*

The 2-DE profiles on the culture filtrate showed that *M. abscessus* and *M. chelonae* secrete about the same range of detectable proteins in the extracellular culture environment (Figure 1). However, a comparison of the two profiles led to an identification of species-specific (unique) and common (shared) proteins (Figure 3). A total of 39 secreted proteins were identified in *M. chelonae*, of which 37 were unique to this species secretome whereas 2 represented shared proteins between the two species. In *M. abscessus*, 34 major proteins were identified, of which 32 were unique to this species secretome.

3.3.1. *Shared secreted proteins*

The two secreted proteins common between the *M. abscessus* and *M. chelonae* secretomes viz. elongation factor Tu (EF-Tu) and Acyl-CoA dehydrogenase (FadE6) are known to play roles in survival or virulence in other *Mycobacterium* species. For instance, EF-Tu is a GTPase which helps in binding of aminoacyl-tRNA to ribosomes during protein biosynthesis and shows up-regulation during hypoxia and high iron conditions *in vitro* [31-33]. This protein is also upregulated during mycobacterial infection of macrophages [33]. It has been shown to be associated with the membrane in *M. leprae* [34] and in *E. coli* during starvation indicating that EF-Tu may have role in the regulation of cell growth and the organism's response to stress such as in response to antimycobacterial therapy [35]. FadE6 belongs to the class of flavoproteins which play important role in the oxidation of fatty acyl-CoA [36]. It uses n-octanoyl-CoA as a substrate

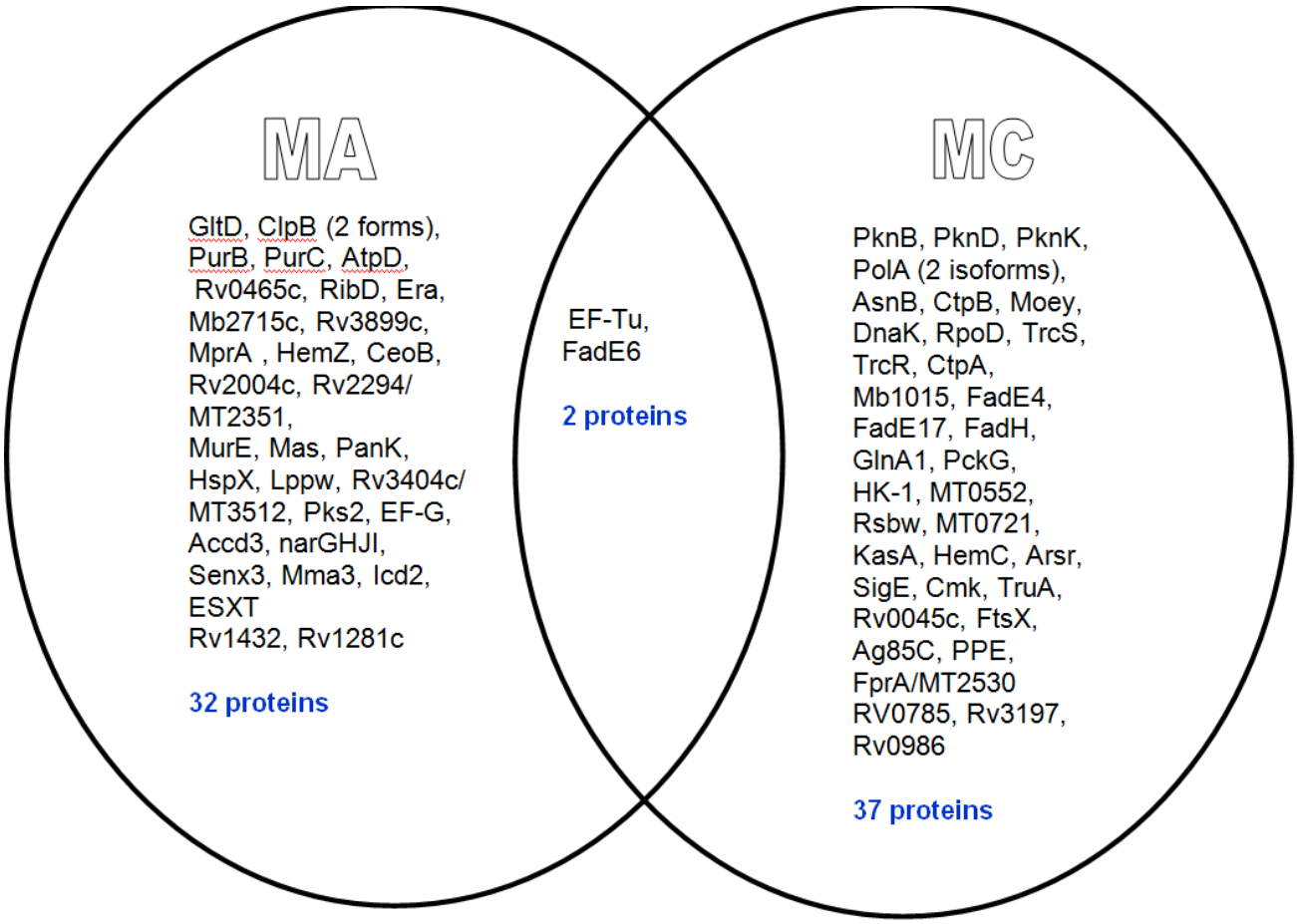

Figure 3. Differential distribution of the secreted proteins in *M. abscessus* versus *M. chelonae*. The two circles in the Venn diagram show the species-specific (unique) proteins for the individual species whereas the overlap region between the two circles shows the proteins common in both species.

Table 1. Identified secretory proteins of *M. abscessus*

Spot no	Protein name	NCBI accession number	MW /pI	Sequence coverage (%)	Number of mass values matched	MOWSE score*	Putative function	Ortholog identified in *M. chelonae ?*
1	Glutamate synthase (GltD)	Q7TVH8	53399/5.52	29	9	32	Metabolism of nitrogen	No
2	Chaperone protein ClpB	Q73T66	92632/5.2	30.3	14	40	Preventing protein aggregation and assisting in the refolding of denatured proteins	No
3	Chaperone protein ClpB	Q73T66	92632/5.2	18	10	25	Involved in protein folding	No
4	Probable acyl-CoA dehydrogenase (FadE6)	B70628	78428/5.27	21	10	25	Acyl-CoA dehydrogenase activity (Fatty acid metabolism)	Yes
5	Adenylosuccinate lyase (PurB)	P71832	51004/5.94	45	23	28	Involved in purine biosynthesis pathway	No
6	ATP synthase subunit beta (AtpD)	B70775	53061/4.86	43	8	27	ATP generation	No
7	Probable transcription regulator (Rv0465c)	CAA17420	53039/6.63	45	23	28	Gene regulation	No
8	Riboflavin biosynthesis (RibD)	P71677	35345/6.40		6	28	Riboflavin biosynthesis	No
9	Bex GTP-binding protein (Era homolog)	P0A562	32440/ 5.76	41	10	33	Essential for cell growth	No
10	Conserved hypothetical alanine, glycine and valine rich protein (Mb2715c)	Q7TY31	27199/5.35	36	10	20	Unknown function	No
11	Hypothetical protein (Rv3899c)	D70599	40763/5.67	28	6	28	Unknown function	No
12	Response regulator protein MprA	Q84BX0	25908/5.29	15	5	27	Signal transduction	No
13	Probable Heme protein (HemZ)	H70710	37121/5.69	27	5	27	Heme biosynthesis	No

Table 1. Identified secretory proteins of *M. abscessus (Continuation)*

14	Probable PurC protein	G70708	32910/5.12	34	10	26	Purine biosynthesis	No
15	K^+ transporter protein (CeoB)	Q7D6R7	24195/5.97	52	7	25	K transport/ drug resistant/ survival in macrophage	No
16	Hypothetical protein (Rv2004c)	B70759	54389/5.89	36	12	21	Unknown function	No
17	Aminotransferase (At) (Rv2294/MT2351)	P63502	44256/5.78	38	7	37	Possible role in nitrogen metabolism	No
18	UDP-N-acetylmuram oylalanyl-D-glutamate --2,6-diaminopimelate ligase (MurE)	P65478	55307/ 6.29	29	12	22	Biosynthesis of cell wall	No
19	Probable dehydrogenase (Rv1432)	Q7D8G3	50445/9.24	23	11	20	Function not known	Yes
20	Putative mycocerosyl (Mas)	Q02277	29808/11.39	66	17	33	Synthesis of branched chain fatty acids	No
21	Type III pantothenate Kinase (PanK)	Q7TW42	29287/5.99	36	12	23	Coenzyme A biosynthesis	No
22	14 kDa antigen HspX	P0A5B8	16217/ 5.00	68	11	28	Antigenic protein	No
23	Lipoprotein precursor (Lppw)	P65305	33381/5.18	23	6	18	An alanine-rich protein found in cell membrane	No
24	Protein Rv3404c (MT3512)	P65073	26498/5.55	52	11	33	Function not known	No
25	Probable polyketide synthase 2 (Pks2)	Q7D4T0	225636/5.29	13	29	21	Lipid metabolism	No
26	Elongation factor G (EF-G)	P0A556	77154/4.96	30	12	32	Protein synthesis	No

Table 1. Identified secretory proteins of *M. abscessus (Continuation)*

27	ABC transporter ATP-binding protein (Rv1281c/ MT1318)	P63395	65253/6.02	21	10	27	Binds with ATP and helps in transport of oligopeptide	Yes
28	Acetyl-CoA carboxylase Accd3 protein (Accd3)	E70783	51740/6.10	15	10	21	Mycolic acid biosynthesis	No
29	Elongation factor Tu	P0A559	43566/5.28	35	8	21	Protein synthesis	Yes
30	Nitrate reductase (narGHJI)	NC_002755	62073/ 6.02	25	11	28	Nitrate assimilation	No
31	Sensor-like histidine kinase (Senx3)	P0A601	44797/ 6.06	19	4	18	Signal transduction	Yes
32	Methoxy mycolic acid synthase 3 (Mma3)	Q8VKH1	34034/5.35	55	16	30	Mycolic acid biosynthesis	No
33	Probable isocitrate dehydrogenase protein (Icd2)	C70848	82498/5.24	18	8	24	Involved in TCA cycle.	No
34	Esat-6 like protein ESXT	O06261	11114/ 5.89	60	4	19	Immunogenic protein	No

Table 2. Identified secretory proteins of *M. chelonae*

Spot no	Protein name	NCBI accession number	MW /pI	Sequence coverage (%)	Number of mass values matched	MOWSE score*	Putative function	Ortholog identified in *M. abscessus* ?
1	Probable serine/threonine -protein kinase (PknK)	Q7TXA9	119373/5.52	8	6	15	Signal transduction	No
2	DNA polymerase I (Pol A)	P46835	99793/5.1	34.5	15	87522	DNA replication	No
3	DNA polymerase I (Pol A)	P0A551	98473/5.0	27.0	9	1.01e+7	DNA replication	No

Table 2. Identified secretory proteins of *M. chelonae (Continuation)*

4	Serine/threonine-protein kinase (PknB)	D70699	66469/ 5.22	19	6	28	Signal transduction	No
5	Probable asparagine synthetase (AsnB)	B70785	72104/6.30	36	15	40	Asparagine biosynthesis	No
6	Cation-transporting P-type ATPase B (CtpB)	P46840	72916/4.79	21	6	26	Transport	No
7	Probable Moey protein	B70741	78133/5.69	12	4	23	Molybdopterin biosynthesis	No
8	Serine/threonine-protein kinase (PknD)	P95308	69502/5.53	15	4	18	Signal transduction	No
9	FAD-binding dehydrogenase (Rv0785)	P71838	61258/9.20	30	11	34	Unknown function	Yes
10	Chaperone protein DnaK	CAA41306	65567/5.16	19	10	28	Heat shock/ chaperonic protein	No
11	RNA polymerase sigma factor D (RpoD)	P0A603	57765/4.72	21	11	25	Transcription initiation	No
12	Probable two component sensor protein TrcS	C70624	54557/5.45	19	4	27	Signal transduction	Yes
13	Probable CtpA protein	D70750	79314/6.69	23	12	36	Copper transport	No
14	Possible conserved exported protein (Mb1015)	Q7U0W8	42715/5.16	46	6	27	Function unknown	No
15	Probable FadE17 protein	B70636	44656/5.98	26	10	32	Lipid metabolism	No
16	Glutamine synthetase (GlnA1)	H70775	53536/5.04	18	4	26	L-glutamine biosynthesis	No

Table 2. Identified secretory proteins of *M. chelonae (Continuation)*

17	Phosphoenolpyruvate carboxykinase [GTP] PckG	P65687	67210/4.92	29	13	40	Carbohydrate metabolism	No
18	Probable acyl CoA dehydrogenase (FadE4)	E70962	63455/5.72	17	7	23	Lipid degradation	No
19	Translation elongation factor Tu (EF-Tu)	A44795	43566/5.28	40	9	30	Protein synthesis	Yes
20	Probable conserved ATP-binding protein ABC transporter (Rv3197)	O53343	49106/6.57	19	6	30	Transporter	Yes
21	Sensor histidine kinase.-HK1	Q7D9B7	52001/6.58	21	5	22	Signal transduction	Yes
22	Hypothetical protein (MT0552)	Q8VKJ9	43068/6.81	24	5	19	Unknown function	No
23	Probable Rsbw protein	H70980	25877/5.39	21	4	26	Acts as an anti-Sigma factor Sig B	No
24	FMN-dependent alpha-hydroxy acid dehydrogenase (MT0721)	Q8VKG1	44442/6.56	15	3	26	Hydroquinone synthesis	No
25	Ketoacyl-ACP synthase A (KasA)	P63455	43289/5.11	9	3	18	Mycolic acid biosynthesis	No

Table 2. Identified secretory proteins of *M. chelonae (Continuation)*

25	Ketoacyl-ACP synthase A (KasA)	P63455	43289/5.11	9	3	18	Mycolic acid biosynthesis	No
26	Acyl-CoA dehydrogenase (FadE6)	C70825	41425/5.29	23	5	32	Fatty acid metabolism	Yes
27	Porphobilinogen deaminase (HemC)	A5TZP0	31919/4.84	16	3	30	Porphyrin biosynthesis	No
28	ABC transporter ATP-binding protein (Rv0986/ MT1014)	O53899	27356/5.65	24	5	22	Role in host interaction	Yes
29	Transcriptional regulator, Arsr family.	Q7DA08	24077/5.13	48	4	33	Regulation of gene expression	No
30	Sigma factor E (SigE)	P66808	24210/4.98	22	2	18	Regulation of gene expression	No
31	Cytidylate kinase (Cmk)	Q81FS2	25227/5.06	35	5	37	Metabolism of nucleotide.	No
32	tRNA pseudouridine synthase A (TruA)	P65847	32600/ 9.98	34	7	60	Formation of pseudouridine	Yes

Table 2. Identified secretory proteins of *M. chelonae (Continuation)*

33	Hypothetical protein (Rv0045c)	E70912	32111/5.24	43	8	27	Possible hydrolase	No
34	Cell division protein FtsX homolog	Q7TX91	32771/8.83	19	4	21	Transporter	No
35	Antigen 85-C precursor (Ag85C)	P0A4V5	36748/5.92	13	3	14	Act as a mycolyl transferase	No
36	Probable two-component regulatory protein (TrcR)	D70624	29191/5.82	14	3	13	Signal transduction	No
37	PPE protein	O06246	19799/5.32	24	3	32	Immunogenic protein	No
38	Probable NADPH - dependent 2,4-dienoyl-CoA reductase (FadH)	Q7U0G7	72827/9.62	16	4	26	Lipid biosynthesis	No
39	Ferredoxin oxidoreductase, α subunit (FprA) (MT2530)	Q7D742	69597/5.29	15	5	23	Possible role in iron metabolism or cytochrome P450 activity	No

*Mowse score: The Mowse score, used in peptide mass fingerprinting, is a "similarity score" derived using a statistical model that calculates the "probability of matching N peaks by random chance".

and acts as a medium-chain acyl-CoA dehydrogenase in *M. tuberculosis* [37]. The shared secreted proteins could be the basis of common virulence characteristics in the two species.

3.3.2. *Species-specific secreted proteins*

It is interesting that *M. abscessus* has the ability to multiply, persist, and cause infection in *in vivo* models [23, 24, 25, Yadav et al unpublished data] whereas *M. chelonae* gets cleared relatively early during infection in these models. It is likely that the unique secreted proteins of *M. abscessus* identified in this study enable these differences in survival and/or virulence of *M. abscessus* inside the host as compared to *M. chelonae*. Specifically, differential secretion of the following major categories of mycobacterial proteins in the two species may be responsible for the species-specific differences in their immunogenicity/virulence and host response characteristics.

3.3.2.1. *ESAT-6 secretion system (ESX) proteins.*

ESX is a type VII secretion system known to exist in Mycobacteria and many other actinobacteria and Gram-positive bacteria. It comprises of 5 paralogs (ESX-1 through ESX-5) in *M. tuberculosis*. Of these, ESX-1 which is responsible for secretion of its prototypical members Early Secreted Antigen 6 kDa (ESAT-6) and 10 kDa culture filtrate protein (CFP-10), has been shown to play an important role in pathogen virulence and survival inside the host. For instance, the ESX-1 components ESAT-6 (ESXA) and CFP 10 (ESXB) form a complex and allow the TB pathogen to survive within macrophages [38]. In contrast, the vaccine strain *M. bovis* BCG which has ESAT-6 deletion in the RD-1 region [39] is unable to persist in the host. Some of the other four paralogous ESX systems are also known to be essential for pathogen growth. In light of these facts about the functional significance of mycobacterial ESX proteins, it is interesting that we detected a putative ESAT-6 like protein ESXT in *M. abscessus* and not in *M. chelonae* culture filtrate. Besides its relevance in conferring differential virulence potential, the ESAT-6 has proven to be a specific diagnostic target for tuberculosis infection such as in QuantiFERON^{R}-TB Gold Test and certain multiplex assays. Likewise, the differentially secreted ESX protein ESXT in *M. abscessus*, could be a potential target for designing species–specific diagnostic assays. In this context, ESXA and ESXB have been previously reported as effective targets for specific diagnosis of *M. abscessus* infection [40]. ELISA-based analysis for these proteins enabled successful diagnosis of infection (abscess) in *M. abscessus*-infected patients (culture positive for *M. abscessus*) but not in the *M. chelonae*-infected patients (culture positive for *M. chelonae*) [40].

3.3.2.2 *Cell wall synthesis or lipid metabolism proteins.*

Several secreted proteins identified in the two species were found to be the homologs of proteins/enzymes involved in biosynthesis or degradation of fatty acids (mycolic acids) and glycolipids, the two key components of the mycobacterial cell wall. Except for the fatty acid oxidizing protein FadE6 which is secreted by both *M. abscessus* and *M. chelonae*, others in this functional category were differentially secreted. For instance, *M. abscessus*-specific secreted proteins were Mycocerosyl (Mas), Polyketide synthase 2 (Pks2), Acyl-CoA carboxylase 3 (AccD3), and Methoxy mycolic acid synthase 3 (MmaA3) whereas those differentially detected in *M. chelonae* secretome were Ketoacyl-ACP synthase A (KasA) and NADPH-dependent 2, 4- Dienoyl-CoA reductase (FadH). In addition, the alanine-rich lipoprotein LppW was detected among the major secreted proteins of *M. abscessus* similar to that observed in TB pathogen [26]. Secretion of the predominantly cell envelope-associated lipoproteins has been ascribed to either shedding (release of acylated lipoproteins) or shaving (proteolytic cleavage) in a recent study on *M. tuberculosis* [27].

It is noteworthy that the cell wall biosynthesis gene *mas* (fatty acid synthase) which is considered unique to pathogenic species of the SGM group [36, 41] was expressed in the *M. abscessus* secretome. Expression of this gene is known to upregulate intracellular growth and survival within macrophages [42]. Another cell wall biosynthesis protein Polyketide synthase 2 (Pks2) is known to be involved in the formation of methyl-branched fatty acyl components of sulfolipids [43] such as sulfolipid-1 (SL-1) and plays a role in establishment of TB infection in human host [44]. Homologs of the proteins involved in biosynthesis of mycolic acids (that form the hydrophobic outer layer of mycobacterial cell wall) showed differential distribution in *M. abscessus* (Acyl-CoA carboxylases 3 (AccD3), Methoxy mycolic acid synthase 3 (MmaA3)) versus *M. chelonae* (Ketoacyl-ACP synthase A (KasA)) secretomes. The FAS pathway protein NADPH-dependent 2, 4- Dienoyl-CoA reductase (FadH) differentially secreted in *M. chelonae* is known to act as a link between the FAS-I and FAS-II pathways [45] .

3.3.2.3. *Stress response proteins*

In response to stresses such as heat, oxidizing conditions, and toxicant exposures, the cells produce a set of proteins loosely referred to as heat shock proteins (HSPs). Some of these proteins act as molecular chaperones which bind with and stabilize proteins at the intermediate stages, including folding, assembly, translocation across membranes, and degradation. In our analysis we found two differentially secreted chaperonic proteins ClpB (*M. abscessus*) and DnaK (*M. chelonae*). ClpB is a heat shock protein [46] of the clp protein family that acts as an ATP-dependent protease. Notably ClpB separated as two distinct spots on the 2-DE gel (Figure 1; Table 1). Since the two spots corresponded to the same protein accession number, these may be the length variants of the same protein. DnaK is a major immunodominant antigen in pathogenic mycobacteria [47, 48] and is found to be

upregulated when cells are subjected to high temperature. It is considered as an inhibitor of heat shock response to enable survival of the cells. Other stress proteins detected in *M. chelonae* but not in *M. abscessus* included an RNA polymerase sigma factor E (SigE), three serine-threonine kinases STPKs (PknB, PknD, and PknK), and the TrcRS two-component system proteins TrcR and TrcS. Additional histidine kinase protein homologs differentially detected in the two species were HK1 (*M. chelonae*) and Senx3 (*M. abscessus*). Differential secretion of the chaperonic and other heat shock proteins in the two species implies their variable potential to survive and respond under physiological stress conditions, which in turn may contribute to their phenotypic diversity in terms of host-pathogen interactions.

3.3.2.4. *Metabolic and respiratory pathways associated proteins.*

This functional category encompassed the largest fraction (32-37%) of the identified secretory proteins in both species (Figure 2). Differential distribution of the proteins of this category in the two species was as follows: *M. abscessus*- Adenylosuccinate lyase (PurB) and Phosphoribosylamidoimidazole- succino-carboxamide synthase (PurC), F1Fo ATP synthase subunit beta (AtpD), Riboflavin biosynthesis enzyme (RibD), Nitrate reductase (narGHJI), Isocitrate dehydrogenase 2 (Icd2), Phosphoenol pyruvate carboxykinase (PckG), TrkA protein (also designated as CeoB), Aminotransferase (At); *M chelonae*- Asparagine synthase B (AsnB), Copper-transporting ATPase (CtpA), FMN-dependent alpha-hydroxy acid dehydrogenase (MmcS), Porphobilinogen deaminase (PBGD), Pseudouridine synthase (TruA), Pseudouridine synthase (TruA).

ABC transporter class of proteins important in various cellular processes with likely role in drug resistance, immunity, and/or pathogenesis [49] were detected in both the secretomes albeit with a differential distribution of the members in *M. abscessus* (Rv1281c) versus *M. chelonae* (Rv0986, Rv3197). Such distribution between the two species implies their role in differentially conferring multiple virulence characteristics to these pathogens.

Notably, the two key nitrogen metabolism pathway enzymes Glutamate synthatase (GltD) and Glutamine synthetase (GlnA1) were differentially detected in *M. abscessus* (GltD) and *M. chelonae* (GlnA1) secretomes. GltD is known to be involved in glutamate synthesis whereas GlnA1 is responsible for the incorporation of ammonia into glutamate to make glutamine at low ammonia concentration [50]. GlnA1 is a major secreted protein in the culture filtrate of *M. tuberculosis* (representing approximately one-third of its total measurable enzyme activity), a feature that is considered highly specific in pathogenic mycobacteria. It is one of the important drug targets in pathogenic mycobacteria considering its crucial roles in pathogen survival inside the phagosome [51]; this occurs via modulation of ammonia levels, which may in turn influence phagosomal pH and phagosome-lysosome fusion and cell wall formation [52]. L-glutamine is considered a major component of the cell wall of pathogenic but not nonpathogenic mycobacteria. Its presence in the *M. chelonae* secretome may be one of the contributing factors to its drug resistance.

3.3.2.5. *Gene regulation and cell division related proteins*

The member proteins of this functional category were differentially distributed between the two species as follows: *M. abscessus* (Elongation Factor G, GTP-binding protein Era homolog); *M. chelonae* (DNA polymerase I or PolA, RNA polymerase sigma factor D (RpoD), Anti-sigma factor (RsbW), Transcription regulatory protein (ArsR), the cell division protein FtsX). Notably PolA separated as two distinct isoforms with distinct accession numbers (Table 2). Being associated with basic cellular functions, these proteins could serve as important drug target candidates.

3.3.2.6 *Immune response-related proteins:*

An estimated 5-6% of the secreted proteins in the two secretomes corresponded to those related to immune response (Figure 3). Differential secretion of these proteins was as follows: *M. abscessus* (ESAT-6, 14 kDa antigen HspX), *M. chelonae* (Antigenic protein 85C (Ag85C), PPE protein).

In addition to the ESAT-6 like protein shared between the two secretomes, *M. abscessus* differentially secreted HspX14 (also known as 16 kDa antigen or HSP 16.3). This protein is required for the growth of mycobacterium in macrophages and is upregulated under anaerobic conditions [53]. It has been proposed to play a negative regulatory role in the multiplication of the TB pathogen during *in vivo* infection [54]. It induces humoral immune response in TB patients and also induces T cell and B cell immune responses in latent infections [55, 56]. In contrast, *M. chelonae* secretome consisted of the major mycobacterial antigens Ag85C and PPE. Ag85C possesses a mycolyltransferase enzyme activity and plays an important role in cell wall biosynthesis and survival in mycobacteria [57]. It forms complex with Ag85A and Ag85B and constitutes a major cell wall component. Secreted protein Ag85B has been used for the diagnosis of tuberculosis [58]. It induces strong ThI response and protects mice from the infection when used for immunization along with Ag85A or with other known mycobacterial antigens [59]. The other secreted protein under this functional category was a homolog of PPE59 (O06246) which belongs to the large PPE multigene family of antigens in mycobacteria. This family constitutes around 10% of the *M. tuberculosis* genome [60]. Members of this protein family are rich in asparagine and glycine and contain multiple repeats of AsnXGlyXGlyXAsnXGly signature as a major polymorphic tandem repeat (MPTR) [60]. Consequently, these proteins possess fewer tryptic cleavage sites making them difficult to detect in global proteomic analysis. Our observation is significant considering that

there are fewer chances of detecting them in culture filtrate as compared to the cell wall fraction. For instance, in a latest otherwise exhaustive global proteomic study on the TB bacillus, only one PPE protein could be detected in the culture filtrate (27). Because of the presence of sequence variation at the C- terminal, it has been suggested that the PPE family proteins are responsible for the antigenic variation in mycobacteria [61]. Differential expression of an extracellular PPE may therefore be responsible for conferring yet unknown differential immunogenic characteristic(s) to *M. abscessus*.

In our recent immunoproteomic study on another species of the MCA complex *M. immunogenum* [28], we detected 4 secreted antigens, including EF-Tu (similar to *M. chelonae* and *M. abscessus*), antigen 85A and CtpA (similar to *M. chelonae*) and OtsB (unlike either species in the current study).

3.4. *Proteins with no known function (orphan proteins)*

In this study, 9 proteins with yet unknown function (orphan proteins) were identified from the secretomes of *M. abscessus* (15% of the total) and *M. chelonae* (11% of the total) (Figure 2). Seven of the orphan proteins are homologs of the *M. tuberculosis* hypothetical proteins namely Rv3899c, Rv2004c, Rv3404c, Mb2715c, Mb1015, MT0552, and Rv0045c (Tables 1 and 2). Two of the orphan proteins viz. FAD-binding dehydrogenase (Rv0785) in *M. chelonae* (Table 2) and a probable dehydrogenase (Rv1432) in *M. abscessus* (Table 1), matched properly named proteins in the database.

4. Concluding remarks

In conclusion, this study provides the first account on the major secretory proteins in the RGM species *M. chelonae* for which the genome sequence is not yet known. Importantly, the study revealed dramatic differences between the secretomes of the two taxonomically close RGM species, *M. abscessus* and *M. chelonae*, which show differences in host immune control and infection response. Majority of the secreted proteins (69 of 73 proteins) showed species-specific distribution implying the significance of the unique sets of secreted factors in conferring the host infection differences to these clinically important mycobacterial species. Further functional studies on the identified species-specific proteins may elucidate their specific underlying role in conferring the virulence and host response (including host immune reaction) potential in the two RGM species and could provide specific targets for development of anti-RGM therapeutics (drugs and vaccines). As an additional impact of the study, the differentially secreted proteins could also be exploited as targets for species differentiation and development of species -specific infection diagnosis tools.

5. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/96/0.

Supplementary Material includes Supplementary file 1: Bioinformatic analysis for protein identification based on Peptide mass fingerprinting and Mascot search.

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