Acta Microbiologica et Immunologica Hungarica 65 (3), pp. 405–418 (2018)
DOI: 10.1556/030.65.2018.033
First published online July 18, 2018

MYCOBACTERIA PRODUCE PROTEINS INVOLVED IN BIOFILM FORMATION AND GROWTH-AFFECTING PROCESSES

JOANA KORABLIOVIENĖ[1,2]*, MYKOLAS MAURICAS[1], ČESLOVA AMBROZEVIČIENĖ[3], MINDAUGAS VALIUS[4], ALGIRDAS KAUPINIS[4], SAULIUS ČAPLINSKAS[5,6] and PAVEL KORABLIOV[1]

[1]Department of Immunology, State Research Institute, Centre for Innovative Medicine, Vilnius, Lithuania
[2]Department of Epidemiological Surveillance, Centre for Communicable Diseases and AIDS, Vilnius, Lithuania
[3]Department of Bacteriology, National Food and Veterinary Risk Assessment Institute, Vilnius, Lithuania
[4]Vilnius University Life Sciences Center, Institute of Biochemistry, Proteomics Center, Vilnius, Lithuania
[5]Faculty of Social Policy, Mykolo Romerio University, Vilnius, Lithuania
[6]Centre for Communicable Diseases and AIDS, Vilnius, Lithuania

(Received: 17 July 2017; accepted: 31 May 2018)

The aim of this study was to determine the effect of mycobacterial proteins on mycobacterial biofilm formation and growth processes. We separated growth-affecting proteins (GEPs) from wild type of *Mycobacterium bovis* and ATCC strain of *Mycobacterium avium subsp. avium*. Our results showed that these mycobacteria-secreted GEPs are involved in biofilm formation, growth stimulatory, and inhibitory processes. Our findings suggest that GEP stimulated *M. avium subsp. avium* growth *in vitro*. Stimulation process was observed in mycobacteria affected with GEP extracted from *M. avium subsp. avium*. We found that both GEPs inhibited the growth of the *M. bovis*. Optical density measurement and visual analysis confirm that GEP plays an important role in biofilm formation process. Most of *M. bovis* GEP are associated with the type VII secretion and general secretion pathways. Our results contribute to a better understanding of the mechanisms underlying mycobacterial biofilm formation and growth-affecting processes and better characterization of mycobacterial proteins and their functions. It is noteworthy that this finding represents the first demonstration of GEP-mediated growth effects on a solid and liquid medium.

Keywords: mycobacterial proteins, mycobacterial biofilms, mycobacterial growth processes

*Corresponding author; E-mail: tamkeviciute.joana@gmail.com

Introduction

The ability of bacteria to communicate and behave as a group for social interactions like a multicellular organism has provided significant benefits to bacteria in host colonization, formation of biofilms, defense against competitors, and adaptation to changing environments [1]. Many bacteria have been found to regulate diverse physiological processes and group activities through a mechanism called quorum sensing (QS) [2].

With the emergence of drug resistance, treating mycobacterial infections is becoming increasingly difficult and hence, looking for newer drug targets, especially those involving QS, is an essential component of mycobacterial research. However, the Gram-positive mycobacteria remain a mystery with no clear evidence known about their QS mechanism [3]. Bioinformatics analysis has revealed the presence of LuxR homologs in *Mycobacterium tuberculosis*, but the experimental supports are lacking [4, 5]. Some of these homologs are ubiquitous across the multiple mycobacterial species and are involved in mycobacterial biofilm formation or persistence, suggesting a possible existence of similar QS mechanisms. Given the fact that biofilm formation is mostly linked with QS regulation [3], the existence of QS in mycobacteria cannot be ruled out. However, this hypothesis needs experimental validation [6].

M. tuberculosis typically forms pellicles at the liquid–air interface in growth media. In recent times, pellicles have been equated to biofilms, because they are held together by extracellular polymeric substance (EPS) produced by the bacterium [7]. *M. tuberculosis* forms biofilms harboring antibiotic-tolerant bacilli *in vitro*, but the factors that induce biofilm formation and the nature of the extracellular material (ECM) that holds the cells together are poorly understood, polysaccharides, proteins, DNA, and lipids are important components of the ECM [8, 9]. However, the composition of the mycobacteria biofilm EPS and the mechanisms governing its formation remain poorly understood [9]. It is known that proteinaceous components include cell surface adhesins, protein subunits of flagella, and pili, secreted extracellular proteins, and proteins of outer membrane vesicles [10]. Better characterization of the proteinaceous components structure, functions, and regulatory circuits controlling biofilm matrix production will provide better understanding of mycobacteria physiological processes, such as host colonization, defense against competitors, and adaptation to changing environments (e.g., antibiotic resistance). Understanding these mechanisms and their controlled social activities may open a new avenue for controlling mycobacterial infections [1, 6, 10]. In this study, we determine the effect of mycobacterial proteins on mycobacteria biofilm formation and growth processes. We characterize these proteins by their gene name, status of existence, molecular

weight, location, function, superfamily, and secretion pathway. Biggest part of these proteins were associated with the type VII secretion (T7S) pathway.

Materials and Methods

Bacterial strains and GEP preparation

Wild type of *Mycobacterium bovis* and ATCC strains of *Mycobacterium avium subsp. avium* (ATCC 15769) and *Mycobacterium terrae* (ATCC 15755) were used throughout these studies. GEPs were extracted from *M. bovis* and *M. avium subsp. avium* and tested *in vitro*: MA GEP – GEP extracted from *M. avium subsp. avium*; MB GEP – GEP extracted from *M. bovis*. Cultures were centrifuged (at 4 °C for 45 min at 4,000 rcf) after 8 and 16 weeks of incubation and passed the filtrate through a low protein-binding 0.2-μm filter (Dismic-13 CP cellulose acetate filters, Advantec, Tokyo, Japan). Concentration of proteins (CP) was quantified by Bradford assay.

Growth of bacteria

Bacterial cultures (10^5 CFU/ml) were transferred on Lowenstein–Jensen medium with pyruvic acid (Becton, Dickinson and Company, http://www.bd.com/europe/regulatory/Assets/IFU/Difco_BBL/244420.pdf). Cultures were affected by Blank Paper Disks (6 mm diameter, Becton, Dickinson and Company) impregnated with GEP and incubated at 37 °C for 8 weeks. At the end of incubation, the number of bacteria colonies was calculated. In total, 100 samples were prepared.

Biofilm formation

To evaluate the effect of GEP on biofilm formation, bacterial cultures were raised in 15-ml screw-capped bottles with 2 ml of culture, 5 ml of media, and 0.5 ml of GEP. At the end of third week of incubation, the caps of bottles were loosened to allow further growth of *Mycobacterium* at the interface. Cultures were incubated at 37 °C for 6 weeks.

Congo red assay and cellulose optical density (OD) measurement

About 2% of Congo red was added to both the control and test samples and continued shaking at 37 °C for 2 h. After 2 h, control and mycobacterium biofilm

cells were centrifuged at 5,000 *g* for 5 min, washed three times with PBS, and then were analyzed visually for Congo red binding. OD measurement was performed at 500 nm.

Filter-aided protein sample preparation (FASP)

Proteins were concentrated on Amicon Ultra-0.5 mL 30 kDa centrifugal filter. Trypsin digestion was performed according to a modified FASP protocol as described by Wisniewski et al. [11]. Briefly, proteins were washed with buffer containing 8 M urea. The proteins were alkylated using iodoacetamide. Buffer was exchanged by washing twice with 50 mM NH_4HCO_3, and proteins were digested overnight with TPCK Trypsin 20233 (Thermo Scientific, USA). Then, peptides were recovered by centrifugation and washed with 20% CH_3CN. Afterward, samples were lyophilized, redissolved in 0.1% formic acid, and analyzed by mass spectrometry (MS).

Liquid chromatography (LC) and MS

The liquid chromatography (LC) separation of trypsin-cleaved peptides was performed with nanoAcquity UPLC system (Waters Corporation, UK). Peptides were loaded on a reversed-phase trap column PST C18 (Waters Corporation) at a flow rate of 15 ml/min using loading buffer of 0.1% formic acid and subsequently separated on HSS-T3 250 mm analytical column (Waters Corporation) in 30-min linear gradient (A: 0.1% formic acid, B: 100% CH_3CN and 0.1% formic acid at a flow rate of 300 nl/min). The nano-LC was coupled online through HDMS Synapt G2 mass spectrometer (Waters Corporation). The data was acquired using Masslynx version 4.1 software (Waters Corporation) in a positive ion mode. LC–MS data were collected using data-independent acquisition mode MSE with online ion mobility separation. Mass range was set to 50–2,000 Da with a scan time set to 0.75 s. A reference compound [Glu1]-Fibrinopeptide B (Waters Corporation) was continuously infused (500 fmol/ml at a flow rate 500 nl/min) and scanned every 30 s for online mass spectrometer calibration purpose.

Data processing, searching, and analysis

Raw data files were processed and searched using ProteinLynx Global SERVER (PLGS) version 3.0.1 (Waters Corporation). *Mycobacterium* protein sequence database from uniprot (September 29, 2017) was used. The following parameters were used to generate peak lists: (1) minimum intensity for precursors

was set to 135 counts, (2) minimum intensity for fragment ions was set to 25 counts, and (3) intensity was set to 750 counts. Processed data were analyzed using trypsin as the cleavage protease, one missed cleavage was allowed, fixed modification was set to carbamidomethylation of cysteines, and variable modification was set to oxidation of methionine. Minimum identification criteria included one fragment ions per peptide, three fragment ions per protein and minimum of two peptides per protein. The false discovery rate (FDR) for peptide and protein identification was determined based on the search of a reversed database, which was automatically generated using PLGS when global FDR was set to 4%.

Statistical analysis

Statistically significant differences between the groups were examined by the Mann–Whitney *U* test and Wilcoxon test; $p < 0.05$ was considered statistically significant, $p < 0.09$ – clear trend.

Results

GEP role in bacterial growth

We found that both GEPs inhibited the growth of *M. bovis in vitro* (Figure 1). The strongest inhibitory process was observed in *M. bovis* affected with MB GEP ($p = 0.030$). Our results indicated that MA GEP stimulated the growth of the *M. avium subsp. avium*, whereas MB GEP inhibited the process (Figure 1). Both GEPs stimulated the growth of the *M. terrae*. The strongest stimulation process was observed in *M. terrae* affected with MA GEP (Figure 1). Statistical significance of results is given in Table I.

GEP role in bacterial biofilm formation

Cellulose is a critical component of mycobacteria biofilms [9], we scraped the biofilm biomaterial and stained biofilms cellulose with Congo red. We observed that higher OD was in samples affected by GEP. OD measurement and visual analysis confirm that GEP plays an important role in biofilm formation process. In samples affected by GEP was enhanced bacterial pellicles, clumps, and aggregates formation process. The most striking OD and visual changes were in the *M. bovis* samples affected by MA GEP (Figure 2). We found that *M. bovis* and *M. avium subsp. avium* affected by GEP have tendency ($p = 0.083$) for higher OD.

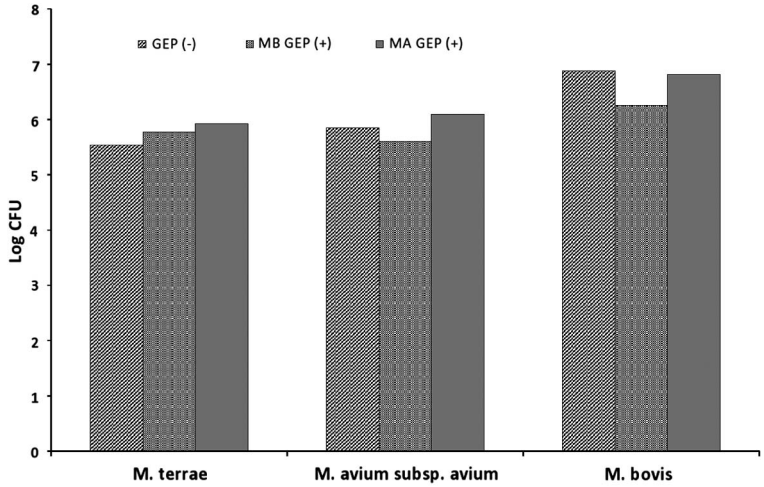

Figure 1. Effect of MB GEP (+) and MA GEP (+) on Log CFU of *M. terrae, M. avium subsp. avium*, and *M.bovis.* Mycobacterial samples without GEP (−) were considered to be control. GEP: growth-affecting protein; MA GEP: GEP extracted from *M. avium subsp. avium*; MB GEP: GEP extracted from *M. bovis*

Table I. Statistical significance of MB GEP and MA GEP on CFU/ml of mycobacteria

Mycobacteria	GEP	Mean rank	Mann–Whitney *U*	Wilcoxon *W*	*z*	*p*
M. avium	–	10.25	2.500	57.500	−1.623	0.121
subsp. avium	MB GEP	5.75				
	–	3.75	4.500	7.500	−1.186	0.273
	MA GEP	7.05				
M. bovis	–	11.5	0.000	55.000	−2.152	0.030
	MB GEP	5.5				
	–	8.0	7.000	62.000	−0.646	0.606
	MA GEP	6.2				

Note: Mycobacterial samples without GEP (−) were considered to be control. GEP: growth-affecting protein; MA GEP: GEP extracted from *M. avium subsp. avium*; MB GEP: GEP extracted from *M. bovis.*

Statistical significance of results is given in Table II. We did not find any statistically significance or tendency in samples with *M. terrae.*

GEP identification

We analyze GEP samples using FASP method and found 22 proteins. We found 20 proteins in MB GEP and two uncharacterized proteins in MA GEP samples (Table III). In samples from *M.terrae*, we did not find any proteins that were identifiable in uniprot database.

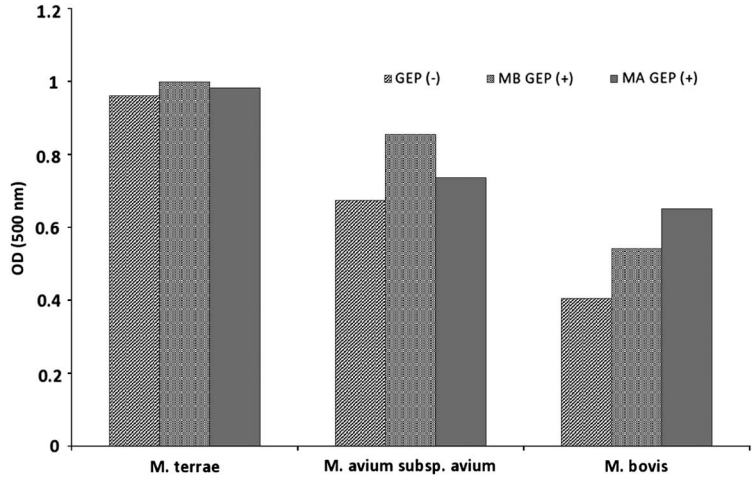

Figure 2. Effect of MB GEP (+) and MA GEP (+) on biofilms scraped from *M. terrae, M. avium subsp. avium*, and *M. bovis* was evaluated by cellulose optical density (OD) measurement. Mycobacterial samples without GEP (−) were considered to be control. GEP: growth-affecting protein; MA GEP: GEP extracted from *M. avium subsp. avium*; MB GEP: GEP extracted from *M. bovis*

Table II. Statistical significance of MB GEP and MA GEP on optical density of cellulose

Mycobacteria	GEP	Mean rank	Mann–Whitney *U*	Wilcoxon *W*	*z*	*p*
M. bovis	–	1.50	0.000	3.000	−1.732	0.083
	MA GEP	4.00				
	–	1.50	0.000	3.000	−1.732	0.083
	MB GEP	4.00				
M. avium subsp. avium	–	1.50	0.000	3.000	−1.732	0.083
	MB GEP	4.00				
	–	1.50	0.000	3.000	−1.732	0.083
	MA GEP	4.00				

Note: Mycobacterial samples without GEP (−) were considered to be control. GEP: growth-affecting protein; MA GEP: GEP extracted from *M. avium subsp. avium*; MB GEP: GEP extracted from *M. bovis.*

Discussion

T7S and general secretion pathways associated with mycobacteria biofilm formation and growth processes

As mentioned above in samples affected by GEP were enhanced bacterial pellicles, clumps, and aggregates formation. The most striking OD and visual changes were in the *M. bovis* samples. We found that *M. bovis* and *M. avium subsp. avium* affected by GEP has tendency for higher OD.

Table III. MB and MA GEP. First 20 proteins were detected in MB GEP and two uncharacterized proteins in MA GEP

No.	Cluster name	Gene name	Status*	kDa	Location	Function	Superfamily	Secretion pathway
1	Major secreted immunogenic protein mpb70	Mpb70	Protein predicted	19	Extracellular protein. Secreted from the mycobacterial cell. Protein is abundantly expressed and secreted into the culture medium [12, 13]	Gene encode preproteins with signal peptides [14]	FAS1 domain	Associated with or are encoded as precursor proteins with typical signal peptides for export through the general secretory pathway. Proteins processed by this system harbor an N terminal signal peptide that is cleaved off as the protein is released on the exterior of the cell. Protein transport through the SecYEG-integral membrane complex [15, 16]
2	Cell surface lipoprotein (fragment)	Mpb83	Protein predicted	20.2	Mycobacterial membrane. Found in the culture filtrate of bacteria grown in liquid culture [17, 18]			
3	Immunogenic protein (fragment)	Mpb64	Protein predicted	24.8	Associated with exocrine protein found in the culture fluid. Secreted protein associated with extracellular region [19, 20]		PdaC/RsiV-like	
4	Membrane protein	B7S04_19330	Protein predicted	42.9	Associated with transmembrane. Integral component of membrane	Membrane proteins involved in the cell envelope. Associated with energy metabolic functions [21]	N/A	N/A
5	ESX-1 secretion-associated protein EspL	B7S05_20825	Protein inferred from homology	12.1	Esx proteins of Esx-1 are generally secreted in different media and not strictly regulated [22, 23]	Proteins (Esx) lack signal peptides and rely on ESX systems for secretion [24]	Nucleoid-associated protein YbaB/EbfC	Associated with the type VII secretion (T7S) pathway [25]
6	PE family protein (uncharacterized)	B7S04_11265	Protein predicted	41.9	Associated with the "cell wall and cell processes" functional category [26]. Detected in culture filtrate [27]	Protein associated with untypical signal peptides [28]	Associated or belong to the same Pfam protein superfamily, designated the EsxAB clan [29]	
7	EsxQ	Mb1595_p3356	Protein predicted	13.0	Associated with ESAT6	Proteins (Esx) lack signal peptides and rely on ESX systems for secretion		
8	ESAT-6-like protein (fragment)	N/A	Protein inferred from homology	10.6	ESAT6 has been reported to be a cell wall protein. Secreted, culture filtrate protein [30, 31]			
9	ESAT-6-like protein	esat6	Protein inferred from homology	9.89				
10	ATP synthase subunit alpha	atpA	Protein inferred from homology	59.2	Inner membrane protein [32]	ATP synthase is reported to be essential in *Mycobacterium* for optimal growth [33]	P-loop containing nucleoside triphosphate hydrolases	
11	Conjugal transfer protein	RN06_4459	Protein predicted	22.1	N/A	Can be associated with several distinct metabolic processes [34]		Type II/IV secretion system protein
12	Probable DNA helicase	BCG_0913c	Protein predicted	59.7	Most of the replisome components are conserved across bacteria [35]	Helicases are motor enzymes that separate/unwind duplex nucleic acid strands [36]		N/A

13	Putative transferase	BCG_1438c	Protein predicted	22.8	N/A	N/A	S-adenosyl-L-methionine-dependent methyltransferase	N/A
14	Uncharacterized protein	BCG_0394c	Protein predicted	22.8	N/A	N/A	Thioesterase/thiol ester dehydrase-isomerase	N/A
15	Methylated-DNA–protein-cysteine methyltransferase	RN06_1638	Protein predicted	5.7	N/A	Involved in the cellular defense against the biological effects of O6-methylguanine and O4-methylthymine in DNA. Can be associated with DNA restoring after damage [37]	Methylated DNA-protein cysteine methyltransferase, C-terminal domain	N/A
16	F420-dependent glucose-6-phosphate dehydrogenase	fgd	Protein inferred from homology	37.5	Can be detected in cell extracts of mycobacteria [38]	Appears to have a role in resistance to oxidative stress, via its consumption of G6P that serves as a source of reducing power to combat oxidative stress in mycobacteria.	Bacterial luciferase-like	Pentose phosphate pathway enzyme [39]
17	GTP 3′,8-cyclase	moaA	Protein inferred from homology	39	MoaA is located on a plasmid [40]	Catalyzes the cyclization of GTP to (8S)-3′,8-cyclo-7,8-dihydroguanosine 5′-triphosphate	Belongs to the radical SAM superfamily	This protein is involved in the pathway molybdopterin biosynthesis, which is part of Cofactor biosynthesis
18	Bacterioferritin	bfrA	Protein inferred from homology	18.3	Iron storage within bacterial cells [41]	Iron-storage protein. Interactive partners of bacterioferritin and ferritin are directly or indirectly involved in *M. tuberculosis* growth, homeostasis, iron assimilation, virulence, resistance, and stresses [42]	Ferritin-like	Associated with the iron related pathways [43]
19	Phenolpthiocerol synthesis type-I polyketide synthase ppsD	ppsD	Protein predicted	19.3	In microbes polyketides are frequently produced in culture after a period of active growth has depleted the substrate [44, 45]	Polyketide synthases are a family of multidomain enzymes or enzyme complexes that produce polyketides structurally diverse secondary metabolites, many of which have antibiotic or anticancer activity, play other roles in the environment other than to defeat microbial competitors [46, 47]	Thiolase-like	Type-I polyketide synthase

Table III. (Cont.)

No.	Cluster name	Gene name	Status*	kDa	Location	Function	Superfamily	Secretion pathway
20	Thioredoxin	B7S05_20990	Protein inferred from homology	12.5	Cytoplasmic protein [48]	Thioredoxins and TrxR have been shown to be involved in reduction of peroxides and dinitrobenzenes and also to detoxify hydroperoxides *in vitro* [49, 50]	Thioredoxin-like	Associated with type III secretion system [51]
1	Uncharacterized protein	RN06_2833	Protein predicted	3.1	N/A	N/A	N/A	N/A
2	Uncharacterized protein	B7S04_00145	Protein predicted	13.1	N/A	N/A	N/A	N/A

Proteinaceous components of the biofilm matrix included secreted extracellular proteins [10]. It is known that Mpb70, Mpb83, Mpb64, EspL, PE family and EsxQ, ESAT-6-like proteins, and ppsD are secreted into the culture medium and can be detectable in culture filtrate. Mpb70, Mpb83, Mpb64, EspL, PE family and EsxQ, and ESAT-6-like proteins are associated with signal peptides. All these proteins are associated with T7S and general secretion pathways (Table III).

Our results indicate that all GEPs inhibited the growth of the *M. bovis*. MB GEP inhibited the growth of the *M. avium subsp. avium*. The strongest inhibitory process was observed in *M. bovis* affected with MB GEP. As discussed above, mycobacteria differently react to their own and closely related slow-growing organism-secreted proteins. The results suggest that MB GEP inhibited *M. bovis* growth, while *M. avium subsp. avium* was stimulated by their own secreted GEP.

There is a lack of information about how mycobacteria responds to their own and closely related, slow-growing organism-secreted proteinaceous compounds. We identified GEP substrate and found that most of the GEP proteins associated with the T7S pathway. Our findings suggest that these mycobacteria-secreted GEP are involved in biofilm formation and growth-affecting processes.

The addition of GEP to liquid culture medium should aid the resumption of normal bacteria growth, which could potentially improve the diagnosis and quantification of mycobacterial infections. They may be involved in mycobacterial reactivation. As well as, these proteins can act as inhibitors. Our results contribute to a better understanding of the mechanisms underlying mycobacterial biofilm formation and growth-affecting processes and better characterization of mycobacterial proteins and their functions.

Acknowledgements

The authors are grateful to Mrs. Rita Viliene for her technical assistance.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Li, Y. H., Tian, X.: Quorum sensing and bacterial social interactions in biofilms. Sensors **12**, 2519–2538 (2012).
2. Waters, C. M., Bassler, B. L.: Quorum sensing: Cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol **21**, 319–346 (2005).

3. Sharma, I. M., Petchiappan, A., Chatterji, D.: Quorum sensing and biofilm formation in mycobacteria: Role of C-di-GMP and methods to study this second messenger. IUBMB Life **66**, 823–834 (2014).
4. Chen, J., Xie, J.: Role and regulation of bacterial LuxR-like regulators. J Cell Biochem **112**, 2694–2702 (2011).
5. Santos, C. L., Correia-Neves, M., Moradas-Ferreira, P. A., Mendes, M. V.: A walk into the LuxR regulators of actinobacteria: Phylogenomic distribution and functional diversity. PLoS One **7**, 46758 (2012).
6. Polkade, A. V., Mantri, S. S., Patwekar, U. J., Jangid, K.: Quorum sensing: An under-explored phenomenon in the phylum Actinobacteria. Front Microbiol **7**, 131 (2016).
7. Ojha, A. K., Baughn, A. D., Sambandan, D., Hsu, T., Trivelli, X., Guerardel, Y., Alahari, A., Kremer, L., Jacobs, W. R., Jr., Hatfull, G. F.: Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. Mol Microbiol **69**, 164–174 (2008).
8. Hans-Curt, F., Wingender, J.: The biofilm matrix. Nat Rev Microbiol **8**, 623–633 (2010).
9. Trivedi, A., Mavi, P. S., Bhatt, D., Kumar, A.: Thiol reductive stress induces cellulose-anchored biofilm formation in *Mycobacterium tuberculosis*. Nat Commun **7**, 11392 (2016).
10. Fong, J. N. C., Yildiz, F. H.: Biofilm matrix proteins. Microbiol Spectr **3**, 10 (2015).
11. Wisniewski, J. R., Zougman, A., Nagaraj, N., Mann M.: Universal sample preparation method for proteome analysis. Nat Methods **6**, 359–362 (2009).
12. Lounatmaa, K., Brander, E.: Immunoelectron microscopic localization of 22 kDa protein antigen in the surface layer of *Mycobacterium bovis* BCG strains. In Lounatmaa, K., Brander, E. (eds): Proceedings of the XIIth International Congress for Electron Microscopy. San Francisco Press Inc., San Francisco, 1990, pp. 894–895.
13. Abou-Zeid, C., Harboe, M., Rook, G. A.: Characterization of the secreted antigens of *Mycobacterium bovis* BCG: Comparison of the 46-kilodalton dimeric protein with proteins MPB64 and MPB70. Infect Immun **55**, 3213–3214 (1987).
14. Wiker, H. G., Harboe, M., Nagai, S.: A localization index for distinction between extracellular and intracellular antigens of *Mycobacterium tuberculosis*. J Gen Microbiol **137**, 875–884 (1991).
15. Wiker, H. G.: MPB70 and MPB83 – Major antigens of *Mycobacterium bovis*. Scand J Immunol **69**, 492–499 (2009).
16. Jungnickel, B., Rapoport, T., Hartmann, E.: Protein translocation: Common themes from bacteria to man. FEBS Lett **6**, 73–77 (1994).
17. Malen, H., Berven, F. S., Softeland, T., Arntzenn, M. O., D'santos, C. S., De Souza, G. A., Wiker, H. G.: Membrane and membrane-associated proteins in Triton X-114 extracts of *Mycobacterium bovis* BCG identified using a combination of gel-based and gel-free fractionation strategies. Proteomics **8**, 1859–1870 (2008).
18. Harboe, M., Wiker, H. G., Ulvund, G., Lund-Pedersen, B., Andersen, A. B., Hewinson, R. G., Nagai, S.: MPB70 and MPB83 as indicators of protein localization in mycobacterial cells. Infect Immun **66**, 289–296 (1998).
19. Harboe, M., Nagai, S., Patarroyo, M. E., Torres, M. L., Ramirez, C., Cruz, N.: Properties of proteins MPB64, MPB70, and MPB80 of *Mycobacterium bovis* BCG. Infect Immun **52**, 293–302 (1986).

20. Yamaguchi, R., Matsuo, K., Yamazaki, A., Abe, C., Nagai, S., Terasaka, K., Yamada, T.: Cloning and characterization of the gene for immunogenic protein MPB64 of *Mycobacterium bovis* BCG. Infect Immun **57**, 283–288 (1989).
21. Gu, S., Chen, J., Dobos, K. M., Bradbury, E. M., Belisle, J. T., Chen, X.: Comprehensive proteomic profiling of the membrane constituents of a *Mycobacterium tuberculosis* strain. Mol Cell Proteom **2**, 1284–1296 (2003).
22. Raman, S., Puyang, X., Cheng, T. Y., Young, D. C., Moody, D. B., Husson, R. N.: *Mycobacterium tuberculosis* SigM positively regulates Esx secreted protein and nonribosomal peptide synthetase genes and down regulates virulence-associated surface lipid synthesis. J Bacteriol **24**, 8460–8468 (2006).
23. Agarwal, N., Woolwine, S. C., Tyagi, S., Bishai, W. R.: Characterization of the *Mycobacterium tuberculosis* sigma factor SigM by assessment of virulence and identification of SigM-dependent genes. Infect Immun **1**, 452–461 (2007).
24. Abdallah, A. M., Gey van Pittius, N. C., Champion, P. A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M., Appelmelk, B. J., Bitter, W.: Type VII secretion – Mycobacteria show the way. Nat Rev Microbiol **5**, 883–891 (2007).
25. Houben, N. G., Korotkov, K. V., Bitter, W.: Take five – Type VII secretion systems of mycobacteria. Biochim Biophys Acta **8**, 1707–1716 (2014).
26. Mazandu, G. K., Mulder, N. J.: Function prediction and analysis of *Mycobacterium tuberculosis* hypothetical proteins. Int J Mol Sci **13**, 7283–7302 (2012).
27. Fishbein, S., van Wyk, N., Warren, R. M., Sampson, S. L.: Phylogeny to function: PE/PPE protein evolution and impact on *Mycobacterium tuberculosis* pathogenicity. Mol Microbiol **5**, 901–916 (2015).
28. de Souza, G. A., Leversen, N. A., Malen, H., Wiker, H. G.: Bacterial proteins with cleaved or uncleaved signal peptides of the general secretory pathway. J Proteomics **2**, 502–510 (2011).
29. Chen, J. M., Zhang, M., Rybniker, J., Basterra, L., Dhar, N., Tischler, A. D., Pojer, F., Cole, S. T.: Phenotypic profiling of *Mycobacterium tuberculosis* EspA point-mutants reveals blockage of ESAT-6 and CFP-10 secretion in vitro does not always correlate with attenuation of virulence. J Bacteriol **24**, 5421–5430 (2013).
30. Majlessi, L., Brodin, P., Brosch, R., Rojas, M. J., Khun, H., Huerre, M., Cole, S. T., Leclerc, C.: Influence of ESAT-6 secretion system 1 (RD1) of *Mycobacterium tuberculosis* on the interaction between mycobacteria and the host immune system. J Immunol **174**, 3570–3579 (2005).
31. Stanley, S. A., Raghavan, S., Hwang, W. W., Cox, J. S.: Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. Proc Natl Acad Sci U S A **22**, 13001–13006 (2003).
32. Lai, E. M., Nair, U., Phadke, N. D., Maddock, J. R.: Proteomic screening and identification of differentially distributed membrane proteins in *Escherichia coli*. Mol Microbiol **52**, 1029–1044 (2004).
33. Sassetti, C. M., Boyd, D. H., Rubin, E. J.: Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol **48**, 77–84 (2003).
34. Lagrost, L., Desrumaux, C., Masson, D., Deckert, V., Gambert, P.: Structure and function of the plasma phospholipid transfer protein. Curr Opin Lipidol **9**, 203–209 (1998).
35. Robinson, A., Causer, R. J., Dixon, N. E.: Architecture and conservation of the bacterial DNA replication machinery, an underexploited drug target. Curr Drug Targets **13**, 352–372 (2012).

36. Matson, S. W., Kaiser-Rogers, K. A.: DNA helicases. Annu Rev Biochem **59**, 289–330 (1990).
37. Tuteja, N., Singh, M. B., Misra, M. K., Bhalla, P. L., Tuteja, R.: Molecular mechanisms of DNA damage and repair: Progress in plants. Crit Rev Biochem Mol Biol **36**, 337–397 (2001).
38. Purwantini, E., Daniels, L.: Purification of a novel coenzyme F420-dependent glucose-6-phosphate dehydrogenase from *Mycobacterium smegmatis*. J Bacteriol **10**, 2861–2866 (1996).
39. Hasan, M. R., Rahman, M., Jaques, S., Purwantini, E., Daniels, L.: Glucose 6-phosphate accumulation in Mycobacteria: Implications for a novel F420-dependent anti-oxidant defense system. J Biol Chem **25**, 19135–19144 (2010).
40. Solomon, P. S., Shaw, A. L., Lane, I., Hanson, G. R., Palmer, T., McEwanl, A. G.: Characterization of a molybdenum cofactor biosynthetic gene cluster in *Rhodobacter capsulatus* which is specific for the biogenesis of dimethylsulfoxide reductase. Microbiology **142**, 1421–1429 (1999).
41. Masse, E., Gottesman, S.: A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc Natl Acad Sci U S A **99**, 4620–4625 (2002).
42. Sharma, D., Bisht, D.: Role of bacterioferritin & ferritin in *M. tuberculosis* pathogenesis and drug resistance: A future perspective by interactomic approach. Front Cell Infect Microbiol **7**, 1–5 (2017).
43. Hameed, S., Pal, R., Fatima, Z.: Iron acquisition mechanisms: Promising target against *Mycobacterium tuberculosis*. Open Microbiol J **9**, 91–97 (2015).
44. Bennett, J. W.: From molecular genetics and secondary metabolism to molecular metabolites and secondary genetics. Can J Botany **73**, 917–924 (1995).
45. Bode, H. B., Bethe, B., Höfs, R., Zeeck, A.: Big effects from small changes: Possible ways to explore nature's chemical diversity. Chembiochem **3**, 619–627 (2002).
46. Staunton, J., Weissman, K. J.: Polyketide biosynthesis: A millennium review. Nat Prod Rep **18**, 380–416 (2001).
47. Adusumilli, S., Mve-Obiang, A., Sparer, T., Meyers, W., Hayman, J., Small, P. L.: *Mycobacterium ulcerans* toxic macrolide, mycolactone modulates the host immune response and cellular location of *M. ulcerans* in vitro and in vivo. Cell Microbiol **7**, 1295–1304 (2005).
48. Aslund, F., Beckwith, J.: The thioredoxin superfamily: Redundancy, specificity, and gray-area genomics. J Bacteriol **181**, 1375–1379 (1999).
49. Zhang, Z., Hillas, P. J., Ortiz de Montellano, P. R.: Reduction of peroxides and dinitrobenzenes by *Mycobacterium tuberculosis* thioredoxin and thioredoxin reductase. Arch Biochem Biophys **363**, 19–26 (1999).
50. Jaeger, T., Budde, H., Flohe, L., Menge, U., Singh, M., Trujillo, M., Radi, R.: Multiple thioredoxin-mediated routes to detoxify hydroperoxides in *Mycobacterium tuberculosis*. Arch Biochem Biophys **423**, 182–191 (2004).
51. Negrea, A., Bjur, E., Puiac, S., Ygberg, S. E., Åslund, F., Rhen, M.: Thioredoxin 1 participates in the activity of the *Salmonella enterica* serovar typhimurium pathogenicity island 2 Type III secretion system. J Bacteriol **191**, 6918–6927 (2009).