

Virulence factors of the *Mycobacterium tuberculosis* complex

Marina A. Forrellad,¹ Laura I. Klepp,¹ Andrea Gioffré,¹ Julia Sabio y García,¹ Hector R. Morbidoni,² María de la Paz Santangelo,¹ Angel A. Cataldi¹ and Fabiana Bigi^{1,*}

¹Instituto de Biotecnología; CICVyA-INTA; Buenos Aires, Argentina; ²Cátedra de Microbiología; Facultad de Ciencias Médicas; Universidad Nacional de Rosario; Rosario, Argentina

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Abbreviations: ABC-transporter, ATP-binding cassette transporter; BMDM, bone marrow-derived macrophages; CFU, colony-forming unit; DAT, di-acylated trehalose; DC, dendritic cell; ECF, extracytoplasmic function; IFN- γ , gamma interferon; LAM, lipoarabinomannane; Lpp, lipoprotein; MAMTs, mycolic acid methyl transferases; MHC-II, major histocompatibility complex class II; MTBC, *Mycobacterium tuberculosis* complex; ORF, open reading frame; PAT, poly-acylated trehalose; PDIM, phthiocerol dimycocerosate; PGL, phenolic glycolipid; *p*-HBADs, *p*-hydroxybenzoic acid derivatives; PIM, phosphatidylinositol mannoside; RD, region of difference; ROS, reactive oxygen species; RNS, reactive nitrogen species; SL, sulfolipid; STM, signature-tagged mutagenesis; TAG, triacylglycerides; TAT, tri-acylated trehalose; TB, tuberculosis; TCS, two-component system; TLR2, Toll-like receptor 2; TDM, trehalose di-mycolates; TMM, trehalose mono-mycolates; TNF- α , tumor necrosis factor-alpha; TraSH, transposon site hybridization; T7SS, type VII secretion system

The *Mycobacterium tuberculosis* complex (MTBC) consists of closely related species that cause tuberculosis in both humans and animals. This illness, still today, remains to be one of the leading causes of morbidity and mortality throughout the world. The mycobacteria enter the host by air, and, once in the lungs, are phagocytated by macrophages. This may lead to the rapid elimination of the bacillus or to the triggering of an active tuberculosis infection. A large number of different virulence factors have evolved in MTBC members as a response to the host immune reaction. The aim of this review is to describe the bacterial genes/proteins that are essential for the virulence of MTBC species, and that have been demonstrated in an in vivo model of infection. Knowledge of MTBC virulence factors is essential for the development of new vaccines and drugs to help manage the disease toward an increasingly more tuberculosis-free world.

Introduction

Members of the genus *Mycobacterium* are characterized by a very complex cell wall envelope that is responsible for the remarkable low permeability of their cells as well as the characteristic differential staining procedure (known as Zhiel-Neelsen acid-fast stain), which specifically stains all members of the genera. Both features are due to the presence of long chain α -alkyl, β -hydroxy fatty acids in their cell wall. The *Mycobacterium* genus is usually separated into two major groups on the basis of their growth rate.

One group includes slow-growing species such as the well-known pathogens *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium leprae* [etiological agents of human tuberculosis (TB), bovine tuberculosis (BTB) and leprosy respectively]; the other group gathers fast-growing species such as *Mycobacterium smegmatis*, which in general are opportunistic or non-pathogenic bacteria.

The *Mycobacterium tuberculosis* complex (MTBC) refers to group of species (*M. tuberculosis*, *Mycobacterium canettii*, *Mycobacterium africanum*, *Mycobacterium microti*, *M. bovis*, *Mycobacterium caprae* and *Mycobacterium pinnipedii*) that are genetically very similar. From those species, *M. tuberculosis* is the most well known member, infecting more than one-third of the world's human population; it is also able to infect animals that have contact with humans. *M. canettii* and *M. africanum*, closely related to *M. tuberculosis*, can also cause human TB and are usually isolated from African patients or African ancestry. *M. bovis* displays the broadest spectrum of host infection, affecting humans, domestic or wild bovines and goats. *M. caprae* has been isolated only from goats. Besides, a laboratory-selected mutant of *M. bovis*, isolated by Calmette and Guérin and known as *M. bovis* var BCG, is the only vaccine used in TB prevention during early childhood. *M. microti* is a rodent pathogen, usually isolated from voles (rodents of the genus *Microtus* and related genera) that can also cause disease in immunocompromised human patients.^{1,2} Finally, *M. pinnipedii* infects seals.³

It has been suggested that MTBC members have evolved from a common ancestor via successive DNA deletions/insertions resulting in the present *Mycobacterium* speciation and their differences in pathogenicity. Genomic analysis has been fundamental for these studies and helped to identify 14 regions (known as regions of difference or RD1–14). These regions, present in the reference laboratory strain *M. tuberculosis* H37Rv,

*Correspondence to: Fabiana Bigi; Email: fbigi@cni.inta.gov.ar
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are absent from the vaccine strain *M. bovis* var BCG; thus, helping to pinpoint chromosomal genes related to pathogenicity. In parallel, six regions, known as H37Rv deletion 1 to 5 (RvD1–5) and *M. tuberculosis* specific deletion 1 (TbD1), are absent from the *M. tuberculosis* H37Rv genome relative to other members. By contrast, *M. canettii* contains all of the RD, RvD and TbD1 regions and it is believed that this is the most closely related genome to that of the bacilli's ancestor. *M. africanum* strains mainly isolated from West Africa lack the RD9 region, whereas those from East Africa have it preserved but lack the RD3. *M. microti* lacks a specific region, named RD^{mic} and the regions RD7, RD8, RD9 and RD10. Some strains that have been isolated from voles missed also part of the RD5 region. The most common *M. bovis* strains, “classical *M. bovis*,” isolated from bovines in Argentina, the Netherlands, United Kingdom and Spain, as well as from humans, showed the greatest number of RD deletions, lacking regions RD4, RD5, RD6, RD7, RD8, RD9, RD10, RD12 and RD13. *M. caprae* is closely related to *M. bovis* except that it contains several nucleotide substitutions in the *gyrB* gene that are not found in other members of the MTBC.⁴ In addition, the lack of the regions RD1, RD2 and RD14 in *M. bovis* var BCG apparently occurred during and after the attenuation process.¹ Even the handling of the original BCG vaccine strain (Pasteur), after being distributed to different centers in the world, has translated into specific mutations present in each of *M. bovis* var BCG strains.

Tuberculosis still remains to be one of the leading causes of mortality throughout the world. The HIV/AIDS pandemic, the deterioration in public health systems in developing countries, and the emergence of multi-drug resistance forms of tuberculosis have contributed further to that spread. The MTBC species infect their mammalian host primarily in the lungs. In this organ, the mycobacteria are engulfed within alveolar macrophages, in which the bacteria are contained in endocytic compartments that can mature to phagosomes. Under normal circumstances, phagosomes are fused to lysosomes and the phagosomal contents are exposed to lysosomal hydrolases, reactive oxygen and nitrogen species that destroy the intracellular bacteria. MTBC species have evolved several mechanisms to circumvent the hostile environment of the macrophage, such as inhibiting phagosome-lysosome fusion and to escape acidic environments inside the phagolysosome.⁵

The infection is normally contained in the lung by formation of granulomas where the activated macrophages and other immune cells surround the site of infection to limit tissue damage and restrict mycobacterial dissemination.^{6,7} Concomitantly, virulent MTBC species have developed strategies to avoid or modulate the immune response in their favor. In the granuloma, some of the bacteria may remain dormant for decades without any active clinical disease (latent tuberculosis). Nevertheless, in any immune-depressing condition the dormant bacteria can become active, replicate and spread into the lung and other tissues.⁷

In recent years, there have been considerable advances in the understanding of the molecular bases of pathogenicity, virulence and persistence of mycobacteria. One significant contribution has been the identification of essential mycobacterial virulence genes.

In particular, the use of transposon mutant libraries in combination with different *in vivo* screening methods has allowed the massive identification of virulence genes and, therefore, the elucidation of mechanisms that the bacilli employ to survive and persist in the hosts. Most of these virulence genes encode enzymes of several lipid pathways, cell surface proteins, regulators and proteins of signal transduction systems. Another group of relevance is the one involved in mycobacterial survival inside the aggressive microenvironment of the host macrophages. It is noticeable that mycobacteria lack classical virulence factors such as toxins, which are typical of other bacterial pathogens, and that many of the virulence genes of MTBC species are also conserved in non-pathogenic mycobacteria. These findings suggest that pathogenic species have adapted their genomes from a free-lifestyle to the intracellular environment with minimal acquisition of exclusive virulence genes.

There are a wide variety of conditions and parameters to define a virulence gene and the discussion about what constitutes a virulence gene is still unsettled. As a consequence, the definitions have not yet being accepted universally by researchers. The existence of opportunistic pathogens and highly susceptible individuals (i.e., immunodeficient ones) further complicates the task to reach precise definitions. Undoubtedly, one requisite to classify a gene as a virulence factor is that its absence attenuates the virulence of the microorganism in an *in vivo* model. However, this criterion comprises a large spectrum of genes, including housekeeping genes that have a function in survival in the host. These housekeeping genes are involved in basic cellular metabolism and are not generally considered as virulence factors.

Taking these concepts into consideration, the present review focuses on those genes/proteins whose inactivation in the mycobacterial genome leads to a measurable loss in pathogenicity or virulence in a validated TB model but fails to impair the bacterial growth in all the standard *in vitro* conditions (excluding stress and starvation) in which the wild-type strain normally replicates. This review also describes genes/proteins such as those required either for expression or transport of virulence factors.

The current review is aimed to update the most recent progress in the identification and characterization of all kind of virulence genes of the MTBC. Previous updates have covered different aspects of the MTBC. The excellent review by Smith fully describes the *M. tuberculosis* pathogenesis and summarizes the contribution of individual genes to the virulence of pathogenic *Mycobacterium* species.⁷ Other revisions have focused on certain aspects of *M. tuberculosis*-host interaction^{5,8} such as defense against host-induced stress,⁹ bacterial carbon metabolism¹⁰ and latent tuberculosis^{11,12}; or particular mycobacterial virulence compounds or genes, such as: proteases,¹³ lipids,¹⁴⁻¹⁶ regulators,¹⁷ sigma factors,¹⁸ secretion systems,^{19,20} among others.

In the present review, the virulence determinants have been divided into the following categories based on their function, molecular features or cellular localization: (1) Lipid and fatty acid metabolism, including catabolism of cholesterol, (2) cell envelope proteins: including cell wall proteins, lipoproteins and secretion systems, (3) proteins inhibiting antimicrobial effectors of the macrophage, including those involved in responses to oxidative

and nitrosative stresses, phagosome arresting and inhibition of apoptosis, (4) protein kinases, (5) proteases, including metalloproteases, (6) metal-transporter proteins, divided into importer and exporters, (7) gene expression regulators, including two component systems, sigma factors and other transcriptional regulators, (8) proteins of unknown function, including PE and PE_PGRS families and (9) other virulence proteins. A brief introduction to each topic is included at the beginning of each section. The information provided here is intended to help readers to better understand the factors that potentially could give rise to a tuberculosis pandemic. Furthermore, it provides and might hopefully reveal a source of potential targets to contain it.

A summary of all virulence genes described in this review is presented in Table 1.

Lipid and Fatty Acid Metabolism

M. tuberculosis is unique among bacterial pathogens in that it displays a wide array of complex lipids and lipoglycans on its cell surface.²¹ These exclusive cell wall lipids are known to play an important role in pathogenesis; therefore, the genes responsible for their biosynthesis, degradation and transport are potential virulence factors that offer new targets for drug design. This section is dedicated to proteins involved in the metabolism and transport of lipids that have been shown to influence mycobacterial pathogenesis and virulence.

Overview of mycolic acids biosynthesis. In order to help understanding the information on mycolic acids related to virulence, we will provide a brief overview of their biosynthetic pathways as shown below.

Mycobacteria are de facto Gram-positive bacteria; however, we can simplify the *M. tuberculosis* cell wall structure describing it as comparable to that of a Gram-negative bacterium. The first macromolecular layer after the peptidoglycan is composed of an heteropolysaccharide composed of arabinan and galactan (thus designated arabinogalactan) to which very long chain α -alkyl β -hydroxy fatty acids (mycolic acids) are esterified. Importantly these mycolic acids are similar in length but different in structure, having either cyclopropanations (*cis* or *trans*) or keto or methoxy groups, creating a number of sub-families. It is also important that besides their structural role, covalently attached to the arabinogalactan, these mycolic acids are also esterified to glycerol and trehalose; in the latter case, trehalose can contain one or two molecules of mycolic acids forming trehalose dimycolates (TDM) and trehalose monomycolates (TMM). Both compounds are present in the cell wall envelope interacting with other complex lipids and lipoglycans as it will be described below. The synthesis of mycolic acids was one of the first of several big surprises, since mycobacteria specialized in the synthesis of fatty acids to make different products. However, mycobacteria keep the essence of the chemistry and use comparable enzymes to the ones used by most of the other bacterial genera. So, while most of the prokaryotes use the fatty acid synthetase (FAS) system to produce fatty acids in the C₁₄-C₁₈ range, mycobacteria use it to make these long chains (up to 86–95 carbon atoms in length) hypothetically starting from a medium length fatty acid.¹⁴ The next surprise was the source of

the latter, which is made by a FAS system homologous to eukaryotic systems.²² In these systems FAS I contains all the catalytic domains in one polypeptide, whereas in the bacterial system (and the mycobacterial mycolic acid synthesis system), known as FAS II, are several enzymes sequentially functioning.

Mycolic acid synthesis apparatus: its relation to virulence. The cytoplasmic synthesis of fatty acids is coordinated with a set of steps in charge of leading them to their final destination out of the cell. If any of the assembly and exporting steps is somehow affected, this will cause an effect in their proper localization.

Different areas of research on tuberculosis have focused the interest on synthesis and export of mycolic acids; in the first place, because several anti-mycobacterial drugs such as isoniazid, ethionamide, isoxyl and thiacetazone affect FAS II enzymes^{23,24} (Belardinelli, personal communication); in the second place, different strategies, mainly based on signature tagged mutagenesis (STM) and specific gene deletion, unmistakably have shown that affecting mycolic acids synthesis or their structure caused alterations in virulence.

Recently Bhatt et al. constructed an *M. tuberculosis* mutant defective in one of the two β -keto acyl synthetases of the FAS II system encoded by the *kasB* gene (Fig. 1).²⁵ Although the gene was clearly not essential (as opposed to the essential *kasA* gene), this deletion resulted in loss of acid-fast staining, alteration of the colony morphology and abolition of classic serpentine growth (traditionally known as “cording” owing to its rope-like form under the microscope). Biochemical analyses revealed that the Δ *kasB* strain produced mycolates with slightly shorter chain lengths, compared with the parental strain. In addition to the distinct phenotypes of this mutant strain, the most remarkable effect of *kasB* disruption was its ability to persist in infected C57BL/6 immuno-competent mice for up to 600 d without causing disease or mortality. These results imply that the *kasB* gene is involved in the pathogenesis of *M. tuberculosis* and that the Δ *kasB* strain could be used as a model to study latent infections.²⁵ Moreover, the extensive identity and similarity between *kasA* and *kasB* (both present in all the mycobacterial species) lead to asking why both genes have been conserved through evolution and how *kasB* expression and/or KasB activity is regulated in order to give biological meaning and relevance to the synthesis of mycolic acids that are just 2–4 carbons shorter than usual.

Another topic in which important advances have been achieved over the last ten years is the mycolic acid methyl transferases (MAMTs), S-adenosyl methionine dependent enzymes which function is to introduce methyl groups, further modified by their conversion to cyclopropane rings, methoxy or keto groups (Fig. 1). As a whole, expression of these enzymes leads to the synthesis of a variety of sub-families for which the studies described below have found amazing and specific roles in spite of very subtle structural differences. Dubnau et al. previously demonstrated that an *M. tuberculosis* mutant with an inactivated *hma* (also known as *cmaA* and more recently as *mmaA4*) gene displayed a profound alteration in its envelope permeability as well as the loss of oxygenated mycolic acids. This mutant, when tested in a mouse (C57BL/6 mice) model of infection by aerosolization, showed an attenuated phenotype, suggesting that

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex

Category	Gen Name	Rv number	Description	Attenuation evidences			
				Model	Result	Complementa- tion	References
Lipids and Fatty Acid Metabolism	<i>kasB</i>	Rv2246	3-oxoacyl-[acyl-carrier protein] synthase 2 kasB	C57BL/6 mice (lda)	Reduced CFUs in organs and lung pathology Increased animal survival	Yes	25
	<i>mmaA4</i>	Rv0642c	Methoxy mycolic acid synthase 4	C57BL/6 mice (lda/iv)	Reduced CFUs in organs	Yes	26
Mycolic acid synthesis	<i>pcaA</i>	Rv0470c	Mycolic acid synthase (cyclopropane synthase)	C57BL/6 mice (iv) ⁺	Failed to persist in the spleens	ND	27
	<i>mymA</i> operon	Rv3083 to Rv3089	Propable Monoxygenase (Hydroxylase)	Activated J774 macrophages and guinea pigs (sc)	Failed to persist in organs Increased animal survival	Yes	
Synthesis of complex lipids	-	Rv2869c	Membrane bound metalloprotease	C57BL/6 mice (lda)	Reduced CFUs in lung	Yes	28
	<i>treS</i>	Rv0126	Trehalose synthase	C57BL/6 mice (iv)	Reduced CFUs in lung Increased animal survival	ND	32
PDIM	<i>pls15</i> <i>pls1</i>	Rv2946c Rv2947c	Probable polyketide synthases	C57BL/6J mice (in) and MAM MH-S	Reduced CFUs	ND	33
	<i>pls10</i>	Rv1660	Possible chalcone synthase	B6D2 F1 mice (lda)	Increased animal survival	ND	34
	<i>pls12</i>	Rv2048c	Probable polyketide synthase	Rabbits (intracisternally)	Reduced CFUs in cerebrospinal fluid and organs	ND	36
	<i>fadD26</i>	Rv2930	Fatty-acid-Coa synthase	C57BL/6J mice (in) and MAM MH-S	Reduced CFUs	ND	37
	<i>fadD28</i>	Rv2941	Fatty-acid-Coa synthase	C57BL/6 mice (iv)	Reduced CFUs	ND	38
	<i>mmpL7</i>	Rv294	Conserved transmembrane transport protein	C57BL/6 mice (iv)	Reduced CFUs	ND	36
	<i>drrC</i>	Rv2938	Probable daunorubicin-dim-integral membrane ABC-transporter	C57BL/6 mice (in) and MAM MH-S	Reduced CFUs	ND	39
	<i>pls5</i>	Rv1527c	Probable polyketide synthase	BALB/c mice (iv)	Reduced CFUs in lung	ND	41
	<i>pls7</i>	Rv1661	Probable polyketide synthase	BALB/c mice (iv)	Reduced CFUs in lung	ND	42
				BALB/c mice (in)	Reduced CFUs in organs	ND	44
				C57BL/6 mice (iv)	Reduced CFUs in lung	ND	41
				C57BL/6 mice (iv)	Reduced CFUs in lung	ND	41
				BALB/c mice (iv)	Reduced CFUs in lung	ND	42
				C57BL/6 mice (lda.)	Reduced CFUs in organs. Increased animal survival	ND	45
				BALB/c mice (lda)	Reduced CFUs in organs	ND	46
				BALB/c mice (lda)	Reduced CFUs in organs	ND	46

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences		
				Model	Result	Complementa-tion
SL	<i>mmpL8</i>	Rv3823c	Probable conserved integral membrane transport protein	C57BL/6 mice (iv)	Reduced CFUs in organs	ND
	<i>fadD33</i>	Rv1345	Possible polyketide synthase	C57BL/6 and B6D2/F1 mice (lda)	Increased animal survival	ND
Others genes related in lipid synthesis	<i>icl1</i>	Rv0467	Isocitrate lyases	BALB/c mice (iv)	Reduced CFUs in liver	Yes
	<i>icl1</i> and <i>icl2</i>	Rv0467 and Rv1915-Rv1916	Isocitrate lyases	C57BL/6 and BALB/c mice (iv). Activated MBMDM	Failed to persist. Increased animal survival Reduced lung pathology	Yes
Catabolism of cholesterol	<i>plcA</i> <i>plcB</i> <i>plcC</i> <i>plcD</i>	Rv2351c Rv2350c Rv2349c Rv1755c	Probable phospholipase C	C57BL/6; IFN- γ ^{-/-} and TNF-R1 ^{-/-} mice (iv). Non-activated and activated BMDM and HBMDM	Reduced CFUs, increased animal survival reduced lung pathology	Yes
	<i>choD</i>	Rv3409c	Putative cholesterol oxidase	BALB/c mice (lda)	Reduced CFUs in organs	Yes
Cell Envelope Proteins	<i>hscC</i>	Rv3568c	3,4-DHSA dioxygenase	C57BL/6 (iv) and mouse peritoneal macrophages	Reduced CFUs	Yes
	<i>igr</i> operon: <i>cyp125</i> <i>fadE28/29</i> - <i>ltp2</i>	Rv3545c Rv3544c-Rv3543c Rv3542c-Rv3541c Rv3540c	Putative cytochrome P450 Acyl coenzyme A dehydrogenases CHPs Probable lipid carrier protein	SCID mice (iv) Guinea pig (a)	Increase survival Modestly reduced CFUs and granulome formation in lungs.	Yes Yes
Cell wall proteins	<i>erp</i>	Rv3810	Exported repetitive protein	C57BL/6 mice (a)	Reduced CFUs	ND
	<i>fbpA</i>	Rv3804	Fibronectin binding protein, mycolyltransferase	MBMDM and BALB/c mice (iv) [†]	Reduced CFUs in macrophages and organs	Yes
Cell wall proteins	<i>mce1</i>	Rv0166 to Rv0174	Mammalian cell entry proteins. Possible lipids ABC-transporters.	BALB/c mice (ip)* THP-1 and J774 macrophages	Reduced CFUs in organs. Reduced CFUs.	Yes ND
	<i>mce2</i>	Rv0586-0594	Cholesterol transporter	BALB/c mice (it) (<i>mce1</i> , 2 and 3 mutants)	Reduced CFUs in organs, reduced tissue pathology and increased animal survival	ND
Cell wall proteins	<i>mce3</i>	Rv1964 to Rv1971	Mammalian cell entry proteins. Possible lipids ABC-transporters.	C57BL/6 mice (lda)	Reduced CFUs and gross lesion in lung. Increased animal survival	ND
	<i>mce4</i>	Rv3501c to Rv3494c	Cholesterol transporter	C57BL/6 mice (lda)	Reduced CFUs and gross lesion in lung. Increased animal survival	ND
Cell wall proteins	<i>mce3</i>	Rv1964 to Rv1971	Mammalian cell entry proteins. Possible lipids ABC-transporters.	C57BL/6 mice (lda)	Reduced CFUs in organs and less tissue pathology in lung. Increased survival	ND
	<i>mce4</i>	Rv3501c to Rv3494c	Cholesterol transporter	C57BL/6 mice (lda)	Reduced CFUs and gross lesion in lung. Increased animal survival	ND

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences			
				Model	Result	Complementation	
	<i>ompATb</i>	Rv0899	Pore-forming protein	THP1 and MBMDM BALB/c mice (lda)	Reduced CFUs in macrophages and organs	ND	107
	<i>hbbA</i>	Rv0475	Heparin binding hemagglutinin protein (Adhesine)	A549 pneumocytes. BALB/c mice (lda) [†]	Reduced adhesion and CFUs in pneumocytes and reduced CFUs in spleen	Yes	111
	<i>pstA1</i>	Rv0930	Inorganic phosphate-ABC transporter	Resting and activated MBMDM	Reduced CFUs	Partial	99
	<i>phot</i>	Rv0820		Resting and activated MBMDM and C57BL/6J mice (iv)	Reduced CFUs in macrophages and lung	Yes	
	<i>caeA</i>	Rv2224c	Carboxylesterase for esters of 3 to 7 carbon atoms	BALB/c mice (lda)	Reduced CFUs and gross pathology in organs	Yes	113
	<i>kefB</i>	Rv3236c	K ⁺ /H ⁺ antiporter, affecting ROS production	BALB/c mice (iv)	Increased survival and weight	Yes	114
	<i>oppABCD</i>	Rv3666c to Rv3663c	Oligopeptide ABC-transporter	C57BL/6 mice (lda)	Moderated reduction in CFUs	Yes	116
Lipoproteins	<i>ctaC</i>	Rv2200c	Citochrome C oxidase unit II	J774 macrophages [†]	Reduced phagosome ROS production	ND	118
	<i>lppX</i>	Rv2945c	Carrier of DIM and antigen	BALB/c mice (lda)	Reduced CFUs in organs in the chronic infection. Increased survival	No	119
	<i>lpqH</i>	Rv3763	Antigen Apoptogenic	MBMDM	Reduced CFUs	ND	127
	<i>lprG</i>	Rv1411c	Cell wall assembly TLR2 agonist	BALB/c mice (ip)*	Reduced CFUs in lung	Yes	138
	<i>lprG-p55</i>	Rv1411c-Rv1410c	Antibiotic efflux pump (P55)	BALB/c mice (ip)*	Reduced bacterial load in spleens	Yes ^a	147
	<i>pstS-1</i>	Rv0934	Inorganic phosphate transport. Antigen and apoptogenic	BALB/c mice (it)*	Reduced CFUs in lung	Yes ^a	145
	<i>lpqY</i>	Rv1235	ABC-transporter (Recycling system of threalose)	J774macrophages*	Reduced CFUs	Yes ^a	157
	<i>modA</i>	Rv1857	Molybdenum ABC transporter	BALB/c and C57BL/6 mice (iv)	Reduced CFUs in organs	ND	157
Secretion system	<i>esxA</i>	Rv3875	Esx-1 component or substrate (C or S)	Mouse peritoneal macrophages	Reduced multiplication	ND	158
	RD1	Rv3868 to Rv3875 and Rv3877	Esx-1 C or S	SCID mice (BALB/c mice background) (lda)	Increased survival	Yes	158
				C57BL/6 mice (lda)	Reduced UFCs in organs.	Yes	42
				CBA/J mice (lda)	Reduced CFUs in ungsand slight increased	ND	170
				BALB/c mice (iv)	Reduced CFUs in lung	ND	170
				Guinea pigs (sc)*	Reduced CFUs in spleen	ND	175
				THP-1 macrophage	Reduced CFUs	No	175
				C57BL/6 mice (a)	Reduced CFUs in organs Total survival	No	175

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences			
				Model	Result	Complementa- tion	
	<i>esxB</i>	Rv3874	Esx-1 C or S	THP-1 macrophage	Reduced CFUs	Yes	187
	<i>espH</i>	Rv3867	Esx-1 C or S	BMDM	Reduced CFUs	NU	186
	<i>espG1</i>	Rv3865	Esx-1 C or S	C57BL/6 mice (lda)	Reduced CFUs in organs	Partial	
				BMDM	Reduced CFUs	NU	186
	<i>espA</i>	Rv3614	Esx-1 C or S	C57BL/6 mice (lda)	Reduced CFUs in organs Total survival	Partial	186
	<i>espC</i>	Rv3615	Esx-1 C or S	C57BL/6 mice (iv)	Reduced CFUs in organs Increased animal survival	Yes	181
	<i>eccCd</i>	Rv3877	Esx-1 C or S	BMDM	Reduced CFUs	Yes	182
	<i>espR</i>	Rv3849	Esx-1 C or S	BMDM	Reduced CFUs	Yes	182
	<i>mycP1</i>	Rv3883	Esx-1 C or S	C57BL/6 mice (iv)	Reduced CFUs in lung	NU	185
				MBMDM	Reduced CFUs	Yes	191
	<i>eccD5</i>	Rv1795	Esx-5 C or S	BALB/c mice (lda)	Reduced CFUs in lung	Yes	202
				MBMDM	Reduced CFUs	Yes	202
	<i>ppe25 to ppe19</i>	Rv1787 to Rv1791	Signal peptidase for lipoproteins	MBMDM	Reduced CFUs	Yes	202
	<i>lspA</i>	Rv1539	Preprotein translocase ATPase	J774 macrophages	Reduced CFUs	NU	123
				CBA/J mice	Reduced CFUs in lung	Yes	
	<i>secA2</i>	Rv1821	Accessory SecA protein	C57BL/6 mice (iv)	Reduced CFUs in organs Increased animal survival	Partial	206
				C57BL/6 -SCID mice (iv)	Increased animal survival	ND	
				C57BL/6 mice (lda)	Increased animal survival Reduced CFUs in organs	ND	272
	<i>secA2</i>	Rv1821	Accessory SecA protein	BMDM from C57BL/6 mice, NOS2 ^{-/-} and pho ^{-/-} mice (lda)	Reduced CFUs	ND	272
Proteins Inhibiting Antimicrobial Effectors of the Macrophage	<i>acr1 (hspX)</i>	Rv2031c	Dormancy-associated protein	MBMDM /THP-1 macrophages	Reduced CFUs	ND	211
	<i>acr2</i>	Rv0251c	Alpha-crystallin (Acr) family of molecular chaperones	C57BL/6 mice (iv)	Reduced weight loss	Partial	217
Oxidative and nitrosative stresses	-	Rv2136c	Likely involved in the synthesis of peptidoglycan	C57BL/6 mice (lda)	Reduced CFUs in organs and reduced gross pathology in lung	ND	114

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences		
				Model	Result	Complementa- tion
	<i>ponA2</i>	Rv3682	Probable transglycosylase and transpeptidase	C57BL/6 mice (Iida)	Moderated reduction in CFUs	ND
	<i>ahpC</i>	Rv2428	Alkyl hydroperoxide reductase c	Guinea pigs (sc) (Antisense)* J774 macrophages	Reduced tissue pathology and CFUs Reduced CFUs	ND ND
	<i>sodC</i>	Rv0432	Superoxide dismutase (SOD) protein	Activated murine peritoneal C57BL/6 mice and iNOS ^{-/-} macrophages	Reduced CFUs	Yes
	<i>mel2</i>	Rv1936 to Rv1941	Bioluminescence-related proteins	Activated J774, MBMDM from infected mice and human PBMC-derived macrophages C57BL/6 and Phox ^{-/-} and iNOS ^{-/-} mice (Iida)	Reduced CFUs Moderated reduction of CFUs in organs	Yes Yes
	<i>katG</i>	Rv1908c	Catalase-peroxidase enzyme	BALB/c mice (iv) Guinea pigs (im) C57BL/6 mice (iv) NOS2 ^{-/-} mice (iv) BMDM from C57BL/6 and NOS2 ^{-/-} mice Activated BMDM NOS2 ^{-/-} Guinea pigs*	Reduced CFUs and increased survival Reduced CFUs in spleen and reduced number of lesion in tissues Reduced CFUs Reduced CFUs Reduced CFUs Reduced CFUs Moderated reduction in CFUs	Yes Yes Yes ND ND Yes
	<i>tpx</i>	Rv1932	Thiol peroxidase	BALB/c mice and MHC class II-knockout mice (fp) Resting and activated BMDM from BALB/c and C57BL/6 mice BALB/c mice (iv)	Reduced bacterial load in organs Reduced CFUs Reduced CFUs and increased animal survival	ND Yes Yes
Phagosome arresting	<i>ndk</i>	Rv2445c	Nucleoside diphosphate kinase	RAW 264.7 macrophages (Antisense) [†]	Reduced CFUs	ND
	<i>ptpA</i>	Rv2234	Low-molecular weight tyrosine phosphatase	THP-1 macrophage BALB/c mice (Iida)	Reduced CFUs Reduced CFUs	Yes Yes
	<i>pe_pgrs30</i>	Rv1651c	Member of the PE family	BALB/c mice (Iida) J774.1 and THP-1 macrophages	Reduced tissue damage Reduced CFUs	ND Yes

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences			
				Model	Result	Complementa- tion	
Inhibition of apoptosis	<i>nuoG</i>	Rv3151	Subunit of the type I NADH dehydrogenase, NADH-1	BALB/c mice (iv)	Increased animal survival and reduced CFUs in organs	Yes	268
	<i>pknE</i>	Rv1743	Serine/threonine kinase E	SCID mice (iv)	Increased animal survival	Yes	275
Protein Kinases	-	Rv3654c-Rv3655c	CHP	U937 apoptotic macrophages	Reduced CFUs	ND	276
	<i>pknD</i>	Rv0931c	Protein kinase D	Brain microvascular endothelial cells (HBMEC)	Impaired invasion	Yes	311
	<i>pknG</i>	Rv0410c	Protein kinase G	BALB/c mice (iv)	Reduced CFUs in brain	ND	
Proteases	<i>mycP1</i>	Rv3883c	Subtilisin-like serine protease.	BALB/c mice (iv)	Increased animal survival	ND	312
	<i>htrA2 (papD)</i>	Rv0983	HtrA-like serine protease and chaperone.	MBMDM /BALB/c (ld a)	Increased animal survival	ND	
Serine proteases	-	Rv3671c	Serine protease	BALB/c, SCID and C57BL/6 mice(iv)	Increased animal survival and less tissue pathology	Yes	328
	<i>digR</i>	Rv2745c	Transcriptional regulator	C57BL/6 mice (lda)	Reduced CFUs in organs and reduced gross pathology in lung	Yes	335
ATP-dependent proteases	<i>zmp1</i>	Rv0198c	Zn ²⁺ Metallo-protease	MBMDM	Reduced CFUs in organs	Yes	334
	<i>rip1</i>	Rv2869c	S2P class of metallo-proteases	J774and RAW264.7 macrophages BALB/c mice (lda)	Reduced CFUs in organs	Yes	340
Metallo-proteases	<i>mpa</i>	Rv2115c	Mycobacterial proteasomal ATPase	THP1 macrophage	No differences in CFU's	No	341
	<i>mbtB</i>	Rv2383c	Iron ABC Transporter	SCID mice (iv)	Reduced survival	No	
Proteasome-associated proteins	<i>pafA</i>	Rv2097c	Mycobacterial proteasomal ATPase	C57BL/6 mice (lda)	Increased CFU's in organs	No	342
	<i>mpa</i>	Rv2115c	Mycobacterial proteasomal ATPase	C57BL/6 mice (a)	Reduced CFUs in organ and reduced tissue pathology	Yes	
Metals-Transporter Proteins	<i>mbtB</i>	Rv2383c	Iron ABC Transporter	C57BL/6 and iNOS ^{-/-} mice	Reduced CFUs in organs and less tissue pathology. Increased animal survival	Yes	349
	<i>mbtB</i>	Rv2383c	Iron ABC Transporter	Resting BMDM from both	Reduced CFUs in organs and pathology	ND	354
Metals-Transporter Proteins	<i>mbtB</i>	Rv2383c	Iron ABC Transporter	MBMDM BALB/c mice (lda)	Reduced CFUs in organs and less tissue pathology. Increased animal survival	Yes	349
	<i>mbtB</i>	Rv2383c	Iron ABC Transporter	Macrophage-like cell line THP-1.	Reduced CFUs and retarded for ability to grow in this cell line.	ND	352

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences		References
				Model	Result	
Metal importers	<i>irtAB</i>	Rv1348-Rv1349	Iron-dependent regulatory protein. Repressor of <i>mtb</i> and <i>mtb-2</i> loci.	THP-1 macrophages. C57B/6 mice (Iida)	Reduced CFUs in macrophages and lung	Yes 354
	<i>ideR</i>	Rv2711		-	Essential	- 356
	<i>mgc</i>	Rv1811	Mg ²⁺ transport P-type ATPase. Mg ²⁺ uptake	HBMDM. BALB/c mice (iv)	Reduced CFUs in macrophages and organs	Yes 357
Metal exporters	<i>ctpC</i>	Rv3270	Zn ²⁺ efflux transporter P-type ATPase	Human macrophages	Reduced CFUs	Yes 358
	<i>ctpV</i>	Rv0969	Cu ²⁺ efflux transporter P-type ATPase	BALB/c mice (Iida) Guinea pigs (Iida)	Increased survival and lower tissue damage in lung Reduced CFUs and lower tissue damage in lung	Yes Yes 359
Gene Expression Regulators	<i>phoPR</i>	Rv0757 to Rv0758	TCS	THP1 macrophages C57BL/6 MICE (Iida)	Reduced CFUs Reduced CFUs in lung	Yes Yes 190
	<i>phoP</i>	Rv0757	Transcriptional regulator	MBMDM BALB/c mice (iv) SCID mice (Iida/iv)	Reduced CFUs Reduced CFUs Improved survival	Yes NU Yes 362 and 364 363
Two component system (TCS)	<i>aprABC</i>	Rv2396abc	Expressed in acidic medium dependent on PhoP	Resting and activated C57BL/6 BMDM	Defects in intracellular replication	Yes 366
	<i>senX3-regX3</i>	Rv0490-Rv0491	TCS	THP-1 macrophages DBA mice	Reduced CFUs Moderate reduced CFUs in lung	ND 367
	<i>senX3</i>	Rv0490	Sensor	BALB/c mice (iv)	Reduced CFUs in lung	Yes 370
	<i>regX3</i>	Rv0491	Transcriptional Regulator	BALB/c mice (iv)	Reduced CFUs in lung	Yes
	<i>dosRS</i> <i>dosT</i>	Rv3133c-Rv3132c Rv2027c	TCS	C57BL/6 MICE (Iida)	Reduced lung pathology. Increased CFUs	Yes 375
	<i>dosR (devR)</i>	Rv3133c	Transcriptional Regulator	C57BL/6 mice (Iida)	Moderate reduction CFUs in lung	ND
				Guinea pigs (Iida)	Reduction CFUs in lung	No
<i>mprAB</i>	Rv0981-Rv0982	TCS	Rabbit (Iida)	Moderate reduction CFUs in lung	ND	
			Guinea pigs (sc)	Reduced lung pathology	NU	378
			BALB/c mice (iv)	Reduced CFUs in lung latent stage	ND	385

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences					
				Model	Result	Complementa-tion			
Sigma factors	<i>sigA</i>	Rv2703	Sigma factor A	C57BL/6 mice (Iida) (Antisense)	Reduced CFUs in lung	ND	390		
				MonoMac6 cells (Antisense)	Reduced CFUs	ND			
	<i>sigC</i>	Rv2069	Sigma factor C	DBA/2 mice (Iida)	Modest reduction of CFUs in lung, increased animal survival and reduced lung pathology	Yes	394		
				SCID mice (Iida)	Modest reduction of CFUs in lung and increased animal survival	Yes			
				Guinea pigs (Iida)	Reduced lung pathology	Yes			
				DBA/2 mice (Iida/iv)	Increased survival and reduced lung pathology	Yes		395	
				Guinea pigs (Iida)	Reduced lung and spleen pathology	ND		396	
				C3H mice (iv)	Increased animal survival	Partial		397	
				BALB/c mice (iv)	Increased animal survival	Partial		398	
				SCID mice	Increased animal survival	No		403	
<i>sigE</i>	Rv1221	Sigma factor E	BALB/c mice (iv)	Reduced CFUs in organs	Yes	402			
			C3H/HeJ mice (a)	Increased animal survival	Partial				
<i>sigF</i>	Rv3286c	Sigma factor F	Unactivated THP-1 and J774 or activated J774 macrophages	Reduced CFUs	Yes	404			
			BALB/c mice (iv)	Increased animal survival	ND	413			
			BALB/c mice (iv)	Reduced CFUs and pathology in tissues	ND	414			
			Guinea pigs (Iida)	Moderated reduction in organ pathology	ND	396			
			J774macrophages	Moderated reduction in CFUs	Partial	416			
			C3H and C57BL/6 mice	Reduced pathology in tissues	Yes	424			
			C3H:He mice	Increased animal survival	Yes	422			
			BMDM from rhesus monkey	Reduced CFUs at late time post infection	Yes				
			<i>sigL</i>	Rv0735	Sigma factor L	BALB/c mice (iv)	Increased animal survival	Yes	426

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences		
				Model	Result	Complementa- tion
	-	Rv0485	Member of the nagC/xylR repressor family	BALB/c mice (iv)	Reduced lung pathology	Yes
				BALB/c mice (iv)	Modestly increased survival	ND
				SCID (iv)	Modestly increased survival	ND
	-	Rv1931c	AraC transcriptional regulator	BALB/c mice (iv) and BMDM from BALB/c mice	Reduced CFUs	Yes
	<i>hspR</i>	Rv0353	Transcriptional repressor	C57BL/6 mice (iv)	Reduced CFUs in organs	ND
	<i>whiB3</i>	Rv3416	Whib-like regulator family	C57BL/6 mice (iv)*	Increased animal survival	ND
	<i>mosR</i>	Rv0348	Mycobacterial operons of survival regulator	BALB/c mice (lda)	Reduced CFUs	No
	<i>virS</i>	Rv3082c	AraC family of transcriptional regulator	BALB/c mice (lda)	Increased animal survival	Partial
	<i>phoY2</i>	Rv0821c	Probable phosphate-transport system transcriptional regulator	Activated J774 macrophage. Guinea pig (sc)	Reduced CFUs	Yes
Proteins of Unknown Function	<i>pe_pgrs33</i>	Rv1818c	PE_PGRS family protein	BALB/c mice (iv)	Reduced CFUs	Yes
The PE/PPE families	<i>pe_pgrs51</i>	Rv3367	PE_PGRS family	J774 and BMDM macrophages [†]	Reduced CFUs	Yes
	<i>ppe46</i>	Rv3018	PPE family	BALB/c mice (iv)	Moderate reduced CFUs in lung	ND
	-	Rv1099c	CHP	C57BL/6J mice (iv)	Reduced CFUs in organs	ND
	-	Rv0573c	CHP	C57BL/6J mice (iv)	Reduced CFUs in organs	ND
	-	Rv0204c	Integral membrane protein	BALB/c mice (iv)	Moderate reduced CFUs in lung	ND
	-	Rv2452c	Hypothetical proteins	BALB/c mice (iv)	Moderate reduced CFUs in lung	ND
Others proteins with unknown function	-	Rv1290c	CHP	CB-17/1cr SCID mice (iv)	Markedly increased survival	ND
	-	Rv1891	CHP	CB-17/1cr SCID mice (iv)	Moderate increased survival	ND
	-	Rv3404c	CHP	CB-17/1cr SCID mice (iv)	Moderate increased survival	ND
	-	Rv1503c to Rv1507c	CHP	BALB/c mice (lda)	Markedly reduced CFUs in organs	Yes
	-	Rv0199	Conserved membrane protein	MBMDM	Reduced CFUs	Yes
	<i>mmpL4</i>	Rv0450c	Conserved membrane transport protein	C57BL/6 x DBA2) F1 mice (a)	Reduced CFUs in organs and increased life survival	No
	-	Rv2136c	Conserved transmembrane protein	C57BL/6 mice (lda)	Markedly reduced CFUs in organs. Decreased gross pathology in lung	No

Table 1. Virulence factor of the *Mycobacterium tuberculosis* complex (continued)

Category	Gen Name	Rv number	Description	Attenuation evidences				
				Model	Result	Complementa- tion		
Other Virulence Factors	RD2	Rv1979c to Rv1982	Region of Difference	C57BL/6 mice (I _{da})	Reduced CFUs in lung and tissue pathology	Yes	467	
	acg	Rv2032	Uncertain	RAW macrophages	Reduced CFUs at late times	Yes	472	
				BALB/c mice (iv)	Reduced CFUs	Yes		
	pckA	Rv0211	Phosphoenolpyruvate carboxykinase	BMDM from BALB/c mice	SCID mice (iv)	Increased animal survival	Yes	473
					BMDM from BALB/c mice [†]	Reduced CFUs	ND	
					BALB/c mice (iv) [†]	Reduced CFUs in spleen	ND	
ptpB	Rv0153c	Tyrosine phosphatase	Activated J774macrophages	Guinea pigs	Reduced CFUs in spleen	Yes	475	
				Reduced CFUs	ND			
hsp22.5	Rv0990c	Novel heat shock protein	BALB/c mice (a)	Reduced CFUs in organs and increased animal survival	Yes	476		

*The mutant used was made in *M. bovis*; [†]in *M. bovis* BCG or [‡]in both *M. tuberculosis* and *M. bovis* BCG. Route of infection: I_{da}, low dose aerosol; a, aerosol; it, intratracheal; iv, intravenous; ip, intraperitoneal; im, intramuscular; sc, subcutaneous. Complementa[†]tion: ND, a complemented strain was not reported by the authors; No, a complemented strain was done but the phenotype was not restored; Yes, a complemented strain was done and the phenotype restored; Yes[‡], the phenotype was restored by the insertion of the entire operon; Partial, a complemented strain was done and the phenotype partially restored; NU: a complemented strain was done but not used by the authors. Abbreviations: CHP, conserved hypothetical proteins; MBMDM, murine bone marrow-derived macrophages; MAM, murine alveolar macrophage; HBMDM, human blood monocyte-derived macrophages.

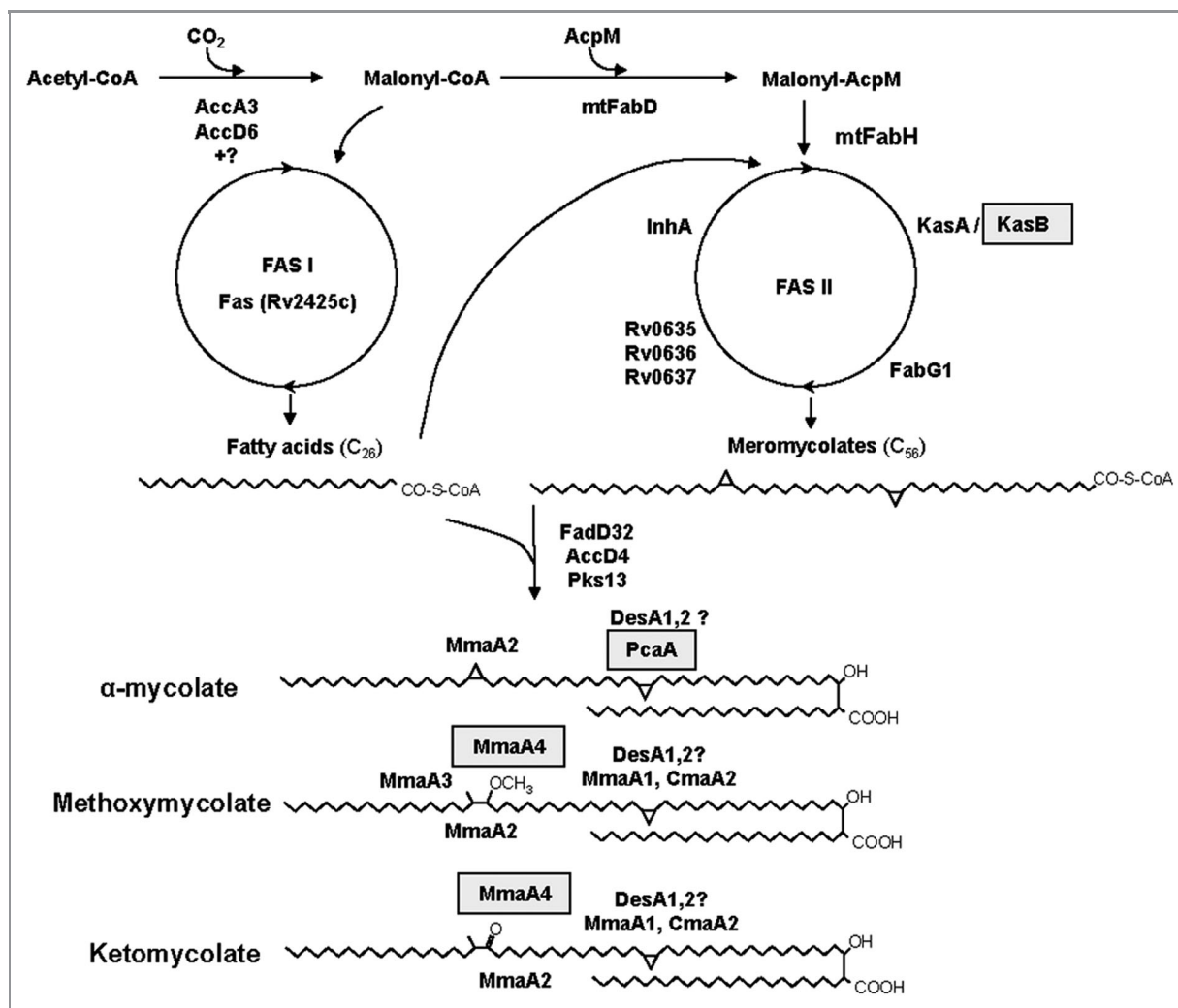


Figure 1. Pathway leading to the biosynthesis of the three types of mycolic acids in *M. tuberculosis*. The genes highlighted in gray were described as virulence factors. KasB, β -keto acyl synthetase; PcaA, Mycolic acid synthase A (cyclopropane synthase); MmaA4, Methoxy mycolic acid synthase 4.

the oxygenated mycolic acids are important in the process of infection.²⁶ Importantly this publication clearly pointed to specific roles of each MAMTs in the biogenesis of mycolic acids and their role in virulence. Continuing this line of research, Glickman et al. demonstrated that the deletion of *pcaA*, whose product catalyzes the proximal cyclopropanation of α -mycolates (Fig. 1), led to loss of cording. They also found that this mutant, despite its normal initial replication, failed to persist and kill infected C57BL/6 mice.²⁷ These results suggest that the site-specific cyclopropane modification of mycolic acids could be an important determinant of the interaction between *M. tuberculosis* and the host. Since this modification of mycolic acids is absent in non-pathogenic mycobacteria, the phenotypes of the Δ *pcaA* strain suggest that the cyclopropyl modification system evolved to mediate principal virulence functions such as interaction with host innate immune receptors. To test this hypothesis, these authors performed further research focused on TDM, a glycolipid that contains cyclopropane modified mycolic acids. In order to explore the role of the Δ *pcaA* phenotype in the recognition by the innate host immune,

this group set out to examine in greater detail the behavior of the Δ *pcaA* mutant during the early stages of infection in the lungs. They infected C57BL/6 mice by aerosol inoculation with the parental or the Δ *pcaA* mutant strains and determined bacterial titers at weekly intervals. Examination at earlier time points showed a drastic initial delay in the growth of the Δ *pcaA* mutant. However, bacterial titers equalized four weeks after infection. The early growth defect of the mutant could be reversed in the complemented strain, demonstrating that the *pcaA* gene was responsible for the observed phenotype. These results indicate that the *pcaA* gene is a temporally restricted determinant of bacterial growth after infection. In addition, the authors showed that purified TDM isolated from the cyclopropane-deficient *pcaA* mutant was hypo-inflammatory for macrophages and induced less severe granulomatous inflammation in mice, demonstrating that the structure of this diffusible glycolipid is critical to its pro-inflammatory activity. Hence, these results imply that the cyclopropyl modification of mycolates on TDM not only modify innate immune recognition but also have a profound effect on the

function of these lipids as important virulence factors of the bacteria.²⁸ Moreover, it demonstrated in an unquestionable way through several very elegant experiments that by subtle structural modifications and different locations (esterified in the cell wall skeleton or loosely attached to the outer membrane) mycolic acids are fundamental not only to preserve cell wall structure and functionality but also to modulate the interaction with the host immune system. This area of research has been recently reinforced by the analysis of the essentiality of each MAMT and the deletion of the seven MAMTs present in *M. tuberculosis*²⁹ demonstrating that none of them is per se essential and that all of them may be deleted although a certain sequence in the deletion has to be followed to allow cellular adjustment of cell permeability and thus survival. This technically challenging work (severely limited by the availability of antibiotic resistance markers) generated a viable strain incapable of making oxygenated or cyclopropanated mycolic acids. This strain showed massive changes in cell appearance such as total loss of acid fastness and susceptibility to detergents. Interestingly, it also displayed a severe attenuation during the first or second week of infection after aerosol infection of mice, depending on whether cyclopropanating enzymes or all the MAMTs were lost. At the same time, the strain generated a marked hyperinflammatory response from the host. This highly valuable work shows the immunomodulatory role of the mycolic acid modifications in the interaction with the host immune system, underscoring the importance of this group of enzymes as molecular targets. It also clearly shows that a novel strategy—targeting non-essential genes that alter the bacilli virulence—is possible.

The mentioned studies on mycolic acid synthesis correlated with others performed on their mechanism(s) of transport and assembly in the mycobacterial cell envelope. In this regard, a gene designated FbpA (part of the antigen 85 complex, Ag85A) has been studied. Belisle et al. identified members of this complex as enzymes responsible for the transfer of mycolic acids to α - α' -trehalose to form α - α' -TMM and α - α' -TDM.³⁰ In a later study, the *fbpA* gene was disrupted in *M. tuberculosis* H37Rv and it was found that it plays a role in the pathogenesis of these bacteria.³¹ The phenotype of this mutant will be further discussed (see the section on Cell wall proteins).

Another *M. tuberculosis* system involved in mycolic acid export that has been found to be important for the cell envelope of the bacteria is the *mymA* operon. To further investigate the function of the genes within this operon, Singh et al. disrupted them in *M. tuberculosis* and analyzed the phenotypes of the obtained mutant strains. The biochemical characterization of the *virS* (the AraC/XylS repressor of the *mym* operon) mutant and the *mymA* mutant showed that both strains displayed reduced contents and altered composition of mycolic acids, along with accumulation of saturated C₂₄ and C₂₆ fatty acids as compared with the wild-type strain. They also found that mutation of these genes impaired the ability of *M. tuberculosis* to survive in activated murine J774 macrophages, but not in resting macrophages, suggesting the importance of the *mymA* operon in protecting the bacterium under unfavorable conditions. Infection of guinea pigs with the mutant and wild-type strains resulted in reduced spleen

bacillary loads of the mutant strains as compared with the wild type in animals at 20 weeks post-infection. However, the bacillary load in lungs of animals infected with the mutants was comparable to that one of animals infected with the parental strain, suggesting that the *mymA* operon is a virulence factor specifically required for growth of *M. tuberculosis* in the spleens of guinea pigs at later stages of the disease³² (see the section on “Other Transcriptional Regulators”).

Finally, a last member of this group of virulence factors is the product of the gene *Rv2869c*, a member of the M50 class of membrane-bound zinc metalloproteases; its disruption translated into various alterations in mycolic acid biosynthesis and phosphatidylinositol mannoside (PIM) composition. These data are consistent with a model in which the *Rv2869c* protease participates in multiple lipid biosynthetic pathways possible through cleavage of membrane bound transcriptional regulators.³³ The constructed mutation resulted in a strain defective for initial replication in the lungs of C57BL/6 mice after aerosol infection and also severely defective for persistence, indicating that although this gene product is not necessary for viability, it is related to virulence.

Even though the synthesis of mycolic acid per se provided a large number of enzymes to be study as virulence factors, the fact that these fatty acids are also acting as diffusible factors in pathogenesis as TDM and TMM led to investigate the pathways involved in the synthesis of trehalose, the major free sugar in the cytoplasm of mycobacteria. In *M. tuberculosis* there are three putative pathways for trehalose synthesis, which is catalyzed by OtsAB, TreS and TreYZ. In an effort to assess their relative contribution to mycobacterial biology, Murphy et al. disrupted five genes from the three pathways: *otsA*, *otsB1*, *otsB2*, *treS* and *treY*. The deletion of the *otsA* gene resulted in marked growth defects of *M. tuberculosis* in vitro and in C57BL/6 mice. Of the *otsB* homologs present in the genome of *M. tuberculosis*, only *otsB2* has a functional role in the pathway and is strictly essential for growth. Inactivation of the TreYZ pathway (Δ *treY*), which can generate trehalose from α -1,4-linked-glucose polymers, had no effect on the growth of *M. tuberculosis* both in vitro and in vivo. The deletion of the *treS* gene altered the late stages of pathogenesis of *M. tuberculosis* in the mouse model, significantly increasing the time to death in chronic infection. The results showed that the OtsAB pathway, which generates trehalose from glucose and glucose-6-phosphate, is the dominant pathway required for *M. tuberculosis* growth. However, since *treS* is the only gene whose deletion resulted in defective growth in vivo but not in vitro, this is the only gene of the three pathways that can be considered a virulence factor of the MTBC according to the criteria taken in this review.³⁴

Synthesis of complex lipids: an overview of their pathways. Although mycolic acids are a remarkable feature shared by all mycobacterial species, *M. tuberculosis* is also characterized by a plethora of complex lipids and glycolipids present in its cell envelope. These lipids and glycolipids are loosely associated to the cell envelope, and thus they can be diffusible factors to modulate the host's immune response or can act as infection stage signals for the pathogen. Cell wall lipids of *M. tuberculosis* containing

multiple methyl-branched fatty acids play important roles in pathogenesis and thus offer targets for new anti-mycobacterial drugs. In *M. tuberculosis*, lipids esterified with multiple methyl-branched fatty acyl substituents include sulfolipids (SL), di- and tri-acylated trehaloses (DAT and TAT), poly-acyltrehaloses (PAT) and phthiocerol dimycocerosates (PDIM). The PDIM structure consists on a long-chain β -diol - phthiocerol - esterified by one type of such long-chain multiple methyl-branched fatty acids called mycocerosic acids. PDIMs constitute major virulence factors of *M. tuberculosis*, in particular during the early step of infection when bacilli encounter their host macrophages. However, although the chemical nature of most of these lipids and glycolipids have been known for some time, only the advent of *M. tuberculosis* H37Rv genome sequence shed some light on their biosynthetic pathways. The exploration of the genome sequence revealed the presence of a large number of genes involved in fatty acid synthesis/modification.²² Intriguingly, it contains a high number of genes with homology to fatty acyl CoA synthases and dehydrogenases as well as a set of genes with homology to polyketide synthases. The latter are multifunctional enzymes that, in other Actinomycetes such as Streptomyces, participate in the synthesis of secondary metabolites like antibiotics, using as building blocks acetate and propionate units among others. Thus, it was tempting to speculate that those *pks* gene products (along with adjacent or neighboring Acyl-CoA synthases and dehydrogenases) could be involved in the synthesis of complex lipids such as mycocerosic acid, sulfolipids, DAT and PATs. In this regard, a seminal work by Kolattukudy's group proposed the hypothetical products of Pks based on the nature of their catalytic domains.³⁵

Biosynthesis apparatus of complex lipids: its relation to virulence. So far, two different approaches have produced information on Pks and accompanying lipid biosynthetic genes. In some cases, the gene of interest was disrupted and the effects of the disruption on the lipid synthesis were assessed. In others, mutants with attenuated virulence were selected using STM.

One of the earliest complex lipids and fatty acids chemically and biochemically characterized is mycocerosic acid, a multiple methyl-branched fatty acid. Seven hypothetical mycocerosic acid synthase (*mas*)-like genes (*msh*) have been identified in the genome of *M. tuberculosis*. One of them, *msh7*, was disrupted in *M. tuberculosis* H37Rv by replacement of an internal segment with a hygromycin resistance gene. This mutant could produce mycocerosic acids but not phthiocerol dimycocerosic acid (PDIM), a molecule biosynthetically derived from the latter. Based on that, the authors suggest that *Msh7* is required for phthiocerol biosynthesis. When the virulence of the mutant was tested infecting intra-nasally C57BL/6J mice, it showed an attenuated phenotype, supporting the hypothesis that PDIMs are important virulence factors.³⁶

In spite of these results, Reed et al. found that an *M. tuberculosis* mutant in the *pks1/15(msh7)* gene was deficient in the production of phenolic glycolipids (PGLs), but not in the synthesis of PDIM. PGLs contain a lipid core composed of phenolphthiocerol esterified by two chains of multiple methyl-branched fatty acids (phthioceranic acids or mycocerosic acids)

and a variable carbohydrate moiety that, according to the mycobacterial species, is composed of one to four *O*-methylated deoxysugars. PGLs are produced by *M. leprae*, *M. kansasii*, *M. bovis*, a few slow-growing mycobacteria and only some strains of *M. tuberculosis*. The work performed by Reed et al. established that the production of PGL in *M. tuberculosis* is associated to the hyper virulent phenotype displayed by a subset of *M. tuberculosis* isolates belonging to the W-Beijing family. These strains showed a "hyper-lethal" behavior in murine infection models. Thus, disruption of *pks1/15* resulted in a strain attenuated in its ability to kill mice following aerosol infection. However, this phenotype was not associated with a defect in multiplication or persistence within the lung or spleen, underlying the exquisite complexity of *M. tuberculosis* pathogenic mechanisms. Thus, the absence of PGLs results in the loss of the hyper virulent phenotype without affecting bacterial load during disease.³⁷ Extending these results, Tsenova et al. tested the role of PGL-tb in a rabbit model of tuberculous meningitis to correlate the severity of disease caused by the *M. tuberculosis* clinical isolates CDC1551 and HN878 or W4, two members of the W-Beijing family strains. Compared with the infection produced by CDC1551, central nervous system (CNS) infection with HN878 or W4 resulted in higher bacillary loads in the cerebrospinal fluid and brain, increased dissemination of bacilli to other organs, persistent levels of tumor necrosis factor- α (TNF- α), higher leukocytosis and more severe clinical manifestations. These authors showed that the disruption of the *pks1/15* gene in HN878 lead to reduced virulence in the rabbit model of infection. Thereby, they concluded that the pathogenic process is associated with the production of PGLs by HN878.³⁸ Altogether these results certainly demonstrate the role of *pks1/15* gene in the synthesis of PGLs and thus in the virulence of pathogenic mycobacteria, whether or not this gene is implicated in PDIMs metabolism of *M. tuberculosis* needs further investigation.

Another *pks* gene whose function was studied is *pks10*, a chalcone synthase-like gene. A $\Delta pks10$ mutant strain displayed the same phenotypes as the *pks1/15* mutant regarding PDIM production and virulence attenuation in C57BL/6J mice, thus it was concluded that *Pks10* is also involved in phthiocerol biosynthesis.³⁶ This scenario becomes more complex and puzzling because a mutant with a deletion in *pks12*, the largest open reading frame in the genome of *M. tuberculosis* H37Rv, was deficient in the synthesis of PDIMs. However, the synthesis of mycocerosic acids was unaffected by this mutation and thus *Pks12* is probably another Pks required for the production of phthiocerol. The growth of this mutant was attenuated in mouse alveolar macrophage and in C57BL/6J mice infected by the intranasal route. Hence, the expression of *pks12* is probably involved in pathogenesis.³⁹

As expected, STM yielded valuable information in connection with the role of pathways leading to the synthesis of PDIM. Two independent STM experiments showed an attenuated phenotype of *FadD26* mutants, a fatty-acid-CoA synthase involved in the biosynthesis of these complex lipids. In one of these experiments, done with *M. tuberculosis* H37Rv, the transposon inserted in the promoter region of *fadD26* and affected the expression of the

downstream *ppsA-ppsE* operon (*Rv2931* to *2935*) encoding a polyketide synthase required for phthiocerol biosynthesis,⁴⁰ thus making the strain deficient in PDIM production. The deletion of the identified genes led to a reduced bacillary load recovered from lungs of intravenously infected mice (C57BL/6) during the initial phase of the infection. In contrast, CFUs recovered from liver and spleen appeared to be unaffected by the mutations. Thus, it was suggested that PDIM would be a virulence factor specifically required for growth of *M. tuberculosis* in the lungs of infected mice.⁴¹ No complementation studies were performed in this experiment; therefore, it is still unknown whether the observed phenotypes of the mutant are solely due to a polar effect on the downstream *pps* operon or reflect a role for FadD26 in these processes. Almost simultaneously, a second independent STM experiment⁴² in *M. tuberculosis* strain 103 yielded two strains with insertions in the *fadD26* gene and in its upstream region. These mutants showed reduced *in vivo* growth when BALB/c mice were infected intravenously. These strains produced little to no PDIMs. Complementation of *fadD26::Tn* produced only 5% of the PDIM level found in the wild type, suggesting that polar effects may have contributed to their corresponding phenotypes.⁴³

In contrast to the results described by Cox et al.,⁴¹ Rousseau et al.⁴⁴ found that CFUs of *fadD26::Tn M. tuberculosis* 103 recovered from lungs and spleens of intra-nasally infected BALB/c mice were reduced as compared with those of the parental strain, raising doubts about the role of PDIM in tissue-specific replication. The reason for the discrepancy between both experiments is not clear but could be a consequence of the differences in the used protocols.

During the STM experimental work, Cox et al. also isolated a Tn5370 insertion in *fadD28*, another fatty-acid-CoA synthase. Its disruption translated in a PDIM production deficiency.⁴¹ As a consequence of these results, Camacho et al. searched for a strain mutant in the *fadD28* gene by screening an insertional mutant library they had previously constructed. This new *fadD28* mutant was also defective in PDIM biosynthesis and its complemented strain had only 15% the level of PDIM produced by the parental strain, which suggests that other genes apart from *fadD28* are responsible for the observed phenotype.

Although the role of *fadD28* in virulence was only evaluated by a high throughput technique and it was not individually tested, its involvement in the production of the pathogenesis associated-PDIM lipid family implies that FadD28 is very likely required for mycobacteria virulence. Nevertheless, further investigation is needed to demonstrate that this gene is indeed a virulence factor.

Along with the identification of the genes involved in the biosynthesis of PDIM, STM experiments brought up the identity of genes whose products were involved in the transport of this important complex lipid. One of such genes encoded for MmpL7, a member of the MmpL protein family that is located within the DIM locus. Insertion mutants in this gene were capable of synthesizing PDIMs but failed to localize them on the cell surface, confirming the role of MmpL7 in the translocation of these lipids across the plasma membrane. Consistent with these results, Domenech et al. showed that MmpL7 is required for normal growth of *M. tuberculosis* H37Rv in aerosol-infected mice.⁴⁵

Another component of the secretion of PDIM is DrrC, which is a member of an ATP-binding cassette (ABC) transporter and works along with MmpL7; thus defects in any of them lead to PDIM accumulation. DrrC was identified in one of the STM transposon searches as being important for *M. tuberculosis* virulence.⁴² Complementation of the DrrC mutant with a copy of the wild-type gene led to full restoration of PDIM production and translocation, demonstrating that the DrrABC transporter, like MmpL7, is essential for PDIM translocation.⁴³ Since the virulence of DrrC was only evaluated by a high throughput technique, further research is required to assert its role in *M. tuberculosis* pathogenesis. Another protein required for the translocation of PDIMs to the outer membrane of *M. tuberculosis* is the lipoprotein Lppx. A mutant in the gene encoding this protein was identified as being highly attenuated in the STM experiment performed by Camacho et al.⁴² Lpp \times will be extensively described below in the section referred to lipoproteins.

A study performed by Rousseau et al.⁴⁶ led to the construction and analysis of a $\Delta pks5$ *M. tuberculosis* mutant. Disruption of this gene, which encodes a mas-like polyketide enzyme, showed no difference in cell envelope lipid composition although it displayed severe growth defects in a mouse model of infection (BALB/c mice). Simultaneously, these researchers also disrupted *pks7*, another mas-like polyketide gene. In contrast with the results obtained for *pks5*, the *M. tuberculosis* $\Delta pks7$ strain was deficient in the production of PDIMs. As expected, the growth of this mutant in BALB/c mice infected via the respiratory route was severely affected.⁴⁶ Thus, a large body of evidence supports the role of PDIM as a virulence factor through the identification of several genes involved in its synthesis. This molecule displays its role in virulence mainly in the early step of infection, when bacilli encounter their host macrophages. Although available information pointed at a mechanism that modulate immune response at the macrophage level, the mechanisms by which the bacilli modulate it are still unknown. A recent study by Astarie-Dequeker et al. in which they used a $\Delta ppsE$ *M. tuberculosis* strain reported that PDIM participates in the receptor-dependent phagocytosis of the tubercle bacilli, as well as in the prevention of phagosomal acidification.⁴⁷ This study demonstrated that this effect was mediated by insertion of PDIM in the host membrane, affecting lipid organization and increasing the efficiency of receptor-mediated phagocytosis of bacilli. These results will clearly help understanding the molecular events through which complex lipids interact with infected host cells, modulating their response to the pathogen's advantage.

Apart from PDIMs, another predominant cell wall lipid is sulpholipid-1 (SL-1), a sulphated glycolipid that has been studied for over 50 years rendering controversial results. Goren et al. showed that SL prevented phagosome-lysosome fusion in cultured macrophages, produced toxic effects on mitochondria by blocking oxidative phosphorylation and suppressing the production of reactive oxygen.^{48,49} Only recently genetic manipulation and the analysis techniques available allowed a detailed characterization of this molecule,^{50,51} which is thought to mediate host-pathogen interactions during infection. However, a direct involvement of SL-1 in mycobacterial virulence has not yet been

established. There are two independent studies that show that MmpL8, a member of a membrane protein family potentially involved in lipid transport in *M. tuberculosis*, is required for SL-1 production. Both studies show that the mutation of the gene encoding MmpL8 in *M. tuberculosis* leads to the accumulation of an SL-1 precursor, indicating that MmpL8 is necessary for an intermediate step in the SL-1 biosynthesis pathway. This precursor, called SL1278, was found accumulate inside the cell, whereas SL-1 was present on the cell surface. These results suggest that the transport and biogenesis of SL-1 are coupled. Both works also showed that *mmpL8* mutants are attenuated for growth in C57BL/6 mice. However, SL-1 per se is not required for establishing infection, since *pk2* mutants that are defective in SL-1 biosynthesis have no obvious in vivo growth defect.^{52,53} These results suggest that either MmpL8 transports other molecules that are implicated in virulence (other than SL-1) or that the accumulation of SL1278 prevents bacterial growth during infection.^{21,45,54}

Other virulence genes involved in fatty acid/lipid metabolism. Although we have offered in the precedent sections an overview of the synthesis of mycolic acids and complex lipids and their roles in the virulence of *M. tuberculosis*, several other pathways are also of importance in the success of this pathogen. Below, we summarize what is currently known about those pathways, and what lies ahead.

FadD33. FadD33 is an acyl-CoA synthase whose gene shows much higher expression in the virulent strain *M. tuberculosis* H37Rv as compared with the avirulent H37Ra strain. Thus, Rindi et al. set out to investigate the potential pathogenic role of this protein. In a first approach, the authors complemented *M. tuberculosis* H37Ra to restore gene expression and studied if this condition conferred any growth advantage to this strain in an infection model of BALB/c mice. They found that, although the growth of the attenuated strain H37Ra was impaired in liver, complementation of this strain with *fadD33* restored bacterial replication in this organ. In a second approach, the *fadD33* gene of *M. tuberculosis* H37Rv was disrupted and the virulence of the generated mutant was evaluated by mouse infection. Again, the absence of the FadD33 protein affected the growth of *M. tuberculosis* in liver but not in lungs or spleen, suggesting that *fadD33* plays a role in *M. tuberculosis* virulence by supporting tissue-specific replication.⁵⁵

Icl1 and Icl2. As an intracellular pathogen, *M. tuberculosis* has to rely on carbon sources obtained from the host's cells to survive. Those metabolic adaptations are important as possible points of intervention through the design of novel drugs. Importantly, the fact that *M. tuberculosis* primarily uses fatty acids instead of carbohydrates during infection is known since the mid 1950s, as reported by Bloch and Segal.⁵⁶ Fatty acids may be used by their catabolism through β -oxidation generating acetyl-CoA, which can be incorporated into the Krebs cycle using an anaplerotic cycle, the glyoxylate cycle. Isocitrate lyase (Icl) is an enzyme that converts isocitrate to succinate in the glyoxylate cycle. This allows bacteria to grow on acetate or fatty acids as sole carbon sources, since the glyoxylate cycle provides a source of carbon that can be further metabolized. In *M. tuberculosis* the glyoxylate cycle

apparently comprises a single gene encoding malate synthase and two genes encoding Icl. The smaller *icl* gene (*icl1*) encodes an enzyme closely related to Icls in other eubacteria, while the larger gene (*icl2*) encodes a protein more homologous to eukaryotic Icls. McKinney et al. have mutated *icl1* gene of *M. tuberculosis* and studied the contribution of this gene to the in vivo metabolism of these bacteria infecting C57BL/6 mice. The mutant initially grew normally in mice, but from the second week onwards the *icl1* mutant was eliminated progressively from the lungs and extrapulmonary organs. In addition, the mutant exhibited wild-type growth in IFN- γ knockout mice and in inactivated macrophages but was killed more rapidly than the parental strain when these macrophages were activated.⁵⁷ Extending these results, Muñoz-Elías and McKinney showed that both prokaryotic- and eukaryotic-like isoforms of the Icl are jointly required for fatty acid catabolism and pathogenesis in *M. tuberculosis*. While mutation of *icl1* or *icl2* had little effect on bacterial growth in a mouse model of infection (C57BL/6 mice) and in both murine BMDM and human blood derived macrophages, the deletion of both genes resulted in complete impairment of intracellular replication and rapid elimination from the lungs.⁵⁸ Additional evidence indicating the importance of Icl includes the observation that *icl* mRNA levels increase in lungs of *M. tuberculosis*-infected C57BL/6 mice as the infection progresses.⁵⁹ These data establish a link between the requirement of Icl and the immune status of the host, suggesting that the in vivo metabolism of *M. tuberculosis* is profoundly influenced by the host response to infection. Thus, the identification and characterization of *M. tuberculosis* glyoxylate cycle not only helped to finally confirm Bloch and Segal's experiments, but also generated at the same time a great deal of information that can be translated into drug design taking advantage of the absence of this cycle in humans. Obviously, more research in this area is warranted considering the proven role of the *M. tuberculosis* Icl enzymes during infection.

PlcA, PlcB, PlcC and PlcD. The *M. tuberculosis* genome has four open reading frames (ORFs) which encode phospholipase C-type enzymes: *plcA*, *plcB*, *plcC* and *plcD*. To study the contribution of these genes to the pathogenesis of *M. tuberculosis*, Raynaud et al. constructed four single mutants of *M. tuberculosis*, each inactivated in one of the *plc* genes, a triple *plcABC* mutant and a quadruple *plcABCD* mutant. Phospholipase C activity was determined in cell extracts of these strains and it was found that all individual mutants had lower enzyme activities compared with the wild-type *M. tuberculosis* 103. Although RT-PCR analysis of the *plc* genes transcripts showed that the expression of these genes was strongly upregulated during the first 24 h of macrophage infection, the triple and quadruple *plc* mutants of *M. tuberculosis* grew normally in these cells. However, the growth kinetics of the triple and quadruple mutants in BALB/c mice revealed that both strains were attenuated in the late phase of the infection, suggesting a role of *plc* in the virulence of *M. tuberculosis*.⁶⁰

Catabolism of cholesterol. As mentioned above, in the intracellular environment, *M. tuberculosis* adapts its metabolism, shifting from one carbohydrate-based to one fatty acid-based.⁵⁸ It has been demonstrated that *M. tuberculosis* uses cholesterol as energy source and for the biosynthesis of the virulence-associated

lipid PDIM.⁶¹ In addition, an increasing number of reports indicated that *M. tuberculosis* metabolizes cholesterol during host infections and that degradation of this sterol contributes to the survival of *M. tuberculosis* in the host.^{61,62} However, while the mutation of genes associated with cholesterol catabolism *choD*, *hsaC* and *mce4* operon attenuated the virulence of *M. tuberculosis*, mutation of *hsd* (encoding a β -hydroxysteroid dehydrogenase) did not reduce *M. tuberculosis* growth inside macrophages or guinea pigs but it was found that this gene is required for bacterial growth on cholesterol as a sole carbon source.⁶³ Therefore, it is plausible to speculate that the in vivo attenuation observed for the above mentioned mutants was due to the toxicity of accumulated metabolites of *M. tuberculosis* cholesterol pathway rather than impairment in the utilization of cholesterol as carbon source. In this regard, several studies focused on *choD*, which encodes a putative cholesterol oxidase. This enzyme catalyzes the oxidation and isomerization of cholesterol to cholestenone (4-cholesten-3-one), which is an initial step in the cholesterol degradation process. The lack of *choD* from *M. tuberculosis* resulted in an impaired replication in C57BL/6 mouse lungs and spleens as compared with the wild-type and complemented strains. Infection of mouse peritoneal macrophages also showed a replication defect in the mutant strain.⁶⁴ Whether the inability of the mutant to use intracellular cholesterol is the reason of the reduced virulence of *choD* mutant needs further investigation.

In the same line of research, HsaC, another enzyme linked to cholesterol catabolism, has been recently studied. *hsaC* encodes a putative extradiol dioxygenase that catalyzes the cleavage of catechols and their analogs. Yam et al. have reported that *M. tuberculosis* produces catechols such as DHSA [3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione] through the metabolism of cholesterol.⁶⁵ In that study, the authors demonstrated that HsaC is a key enzyme in the cholesterol catabolic pathway. Importantly, an *M. tuberculosis* mutant deleted in the *hsaC* gene completely failed to grow on cholesterol and killed SCID mice in more time than the wild-type and complemented strains. Moreover, bacterial loads of lungs from guinea pigs infected with the mutant strain were modestly reduced as compared with those of the animals infected with the wild-type and complemented strains. This attenuated phenotype in lungs correlated with reduced granulome formation in this organ.

Finally, another player identified in the metabolism of cholesterol by *M. tuberculosis* is the *igr* operon, which contains genes for a putative cytochrome P450 (*cyp125*), two acyl coenzyme A dehydrogenases (*fadE28/29*), two conserved hypothetical proteins (*Rv3541c*, *Rv3542c*), and a probable lipid carrier protein (*ltp2*). Based on the homology with a *Rhodococcus* cholesterol-catabolic locus and the genetic interaction with genes encoding the cholesterol transporter *Mce4*,⁶⁶ it has been proposed that the *igr* operon encodes enzymes of cholesterol catabolism. Consistent with this proposed role for *igr* operon, Chang et al. have demonstrated that the lack of this locus inhibits the growth of *M. tuberculosis* in the presence of cholesterol and that this growth inhibition was not due to inability of the bacteria to use the steroid as carbon source but to accumulation of toxic intermediate generated in the initial steps of its degradation.⁶⁷ A

M. tuberculosis deleted in *igr* operon replicated less in C57BL/6 mouse lungs and could not colonized the spleens, as compared with the wild-type H37Rv strain. Remarkably, the deletion of the *mce4* operon from this mutant strain rescued this in vivo attenuated phenotype, indicating that in the absence of cholesterol uptake the *M. tuberculosis igr* mutant restored its full virulence.⁶⁷ Based on their finding, the authors proposed that the in vivo attenuation of *igr* mutant was most likely due to its inability to metabolize cholesterol fully.

Thus, all the information currently available reinforces the concept of the importance of cholesterol metabolism for a successful mycobacterial infection, although the relative contribution of the partial degradation of this compound as carbon source seems to be of lesser importance than the total metabolism to avoid accumulation of toxic intermediates. As cholesterol is a major component of human cell membranes and its concentration there modulates membrane fluidity and engulfment processes, it is clear that this area of research will be quite active in the near future. Moreover, cholesterol is a precursor of steroidal sex hormones, which (among other functions) control immune response. Recent studies have reported that the changes of these hormones correlate to the severity of TB in humans⁶⁸; thus it is not farfetched to assume that we are just starting to learn about a new way by which *M. tuberculosis* became a successful pathogen adapted to humans.

In conclusion, as it was mentioned above, *M. tuberculosis* is exclusive among bacterial pathogens in that it displays a large array of complex lipids on its cell envelope. Indeed, the lipid content of the cell envelope of mycobacteria may represent up to 40% of the cell dry mass.⁶⁹ This exclusive feature reflects the large proportion of the mycobacterial genome devoted to lipid and fatty acid metabolism: about 250 genes are involved in lipid metabolism in *M. tuberculosis*, vs. only 50 such genes in *E. coli*, which has a similar genome size.⁷⁰ Many of these lipids are documented to be important virulence factors of the bacteria and the mechanisms by which they play relevant roles during infection are diverse. Some lipids present in the mycobacterial cell envelope constitute key ligands for the host cell receptors, allowing molecular docking between the host phagocytes and the mycobacteria, leading to cell invasion. Once inside phagocytic cells, *M. tuberculosis* avoids lysosomal fusion and acidification, residing in an immature phagosome.^{71,72} It was found that some mycobacterial lipids such as TDMs, Man-LAM and PDIMs^{47,73,74} play important roles in intracellular trafficking and the vacuole maturation arrest by *M. tuberculosis*. In addition, some mycobacterial lipids modify host cell signaling, affect the secretion of cytokines necessary for protection, participate in the inflammation process during tuberculous infection, or are recognized as antigens by the adaptive immune system. Apart from being involved in these mechanisms of host-cell interactions, some lipids also influence virulence because they display a structural function as being part of the mycobacterial cell wall, which presents a notably low permeability to nutrients and antibacterial drugs. This feature slows down the growth of the bacteria and makes disease caused by pathogenic species difficult to treat. As a consequence of these mechanisms of action, many mycobacterial lipids, and therefore

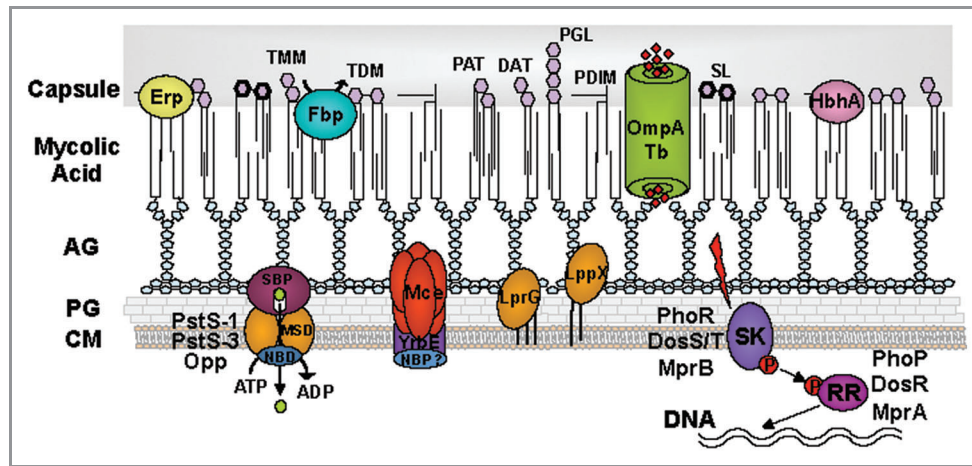


Figure 2. Schematic representation of cell envelope proteins embedded in the cell wall of the of *M. tuberculosis* complex. Proteins like Erp, Fbp, HbhA and the porin OmpATb are exposing to the surface. The ABC transporters: Pst-1, Pst-3, Opp and the Mce proteins consist in the substrate binding proteins (SBP), the permeases (MSD) and the nucleotide binding protein (NBD). The two component systems: PhoR-P, DosS/T-R and MprAB consist in the sensor kinase (SK) that senses the stimuli and the response regulator (RR), which induce the gene transcription. AG, arabinogalactan; PG, peptidoglycan; CM, cytoplasmic membrane; TMM, TDM, trehalose mono and di mycolate; DAT, PAT, di and poli acyl trehalose; PGL, phenolic glycolipid; PDIM, phthiocerol dimycocerosate; SL, sulfolipid.

the genes encoding proteins involved in their metabolism, constitute very important virulence factors of the pathogenic species. However, some lipids, such as mycolic acids and PIMs, present only a few genes involved in their metabolism whose mutation affect the pathogenesis of the bacteria. The reason of this feature is that they are essential for mycobacterial viability and thus the variations in their structure have to be minimal. On the other hand, there are other lipids, such as PDIMs, which are not required for viability but display an important function during the infection process. As a consequence, there are no clinical isolates lacking PDIMs, but it was reported that *M. tuberculosis* H37Rv is highly prone to losing the ability to synthesize PDIMs during extended periods of in vitro culture.⁷⁵ Due to this feature there is a large list of genes involved in PDIMs metabolism that influence the pathogenesis of the bacteria when they are mutated. Finally, there is a third group of lipids, such as PGLs and SLs, which are not required for virulence per se, but influence the development or clinical signs of the disease. This is the explanation for the different proportions of genes involved in the biosynthetic pathways of the diverse lipids described to date as virulence factors. However, further research is needed to identify new genes important in the disease process, since many proteins described as virulence factors by high throughput techniques were not yet individually tested.

Cell Envelope Proteins

As mentioned above, the cell wall, an intricate structure of complex lipids and proteins, is the hallmark of mycobacteria. Its “core” is comprised of peptidoglycan covalently bound to a linear galactofuran, in turn attached to several strands of a highly branched arabinofuran joined to mycolic acids that are very long chain α -alkyl β -hydroxy fatty acids. These lipids are perpendicularly oriented to the plane of the membrane and provide a special

barrier responsible for many of the physiological and disease-inducing aspects of mycobacteria. Intercalated within this “core” are the PDIMs, TDM, SLs and PIMs.⁵⁰ In turn, phosphatidylinositol mannosides, lipomannan (LM) and lipoarabinomannan (LAM) are anchored to the plasma membrane and extend to the exterior of the cell wall. The most external layer of mycobacteria is the capsule, a structure that has been recognized and described later than other cell envelope components.⁷⁶ The capsule contains polysaccharides and minor amounts of inner lipids. There are a number of proteins embedded in this matrix whose function is related to the synthesis and maintenance of the cell wall and that are also responsible for the adhesion, infection, transport of solutes (porins) and survival of mycobacteria in the host cells (Fig. 2).

In this section, we review (1) proteins that are either localized entirely in the cell wall or with a trans-membrane domain exposed toward it, (2) lipoproteins and (3) secretion systems.

Cell wall proteins. Proteomic studies of the mycobacteria cell wall have identified more than five hundred proteins in this cellular structure, including secreted cell wall proteins and lipoproteins.^{77,78} Most of these putative cell wall proteins are involved in cell wall processes and intermediary metabolism. Among these identified putative cell wall proteins, more than 5% have been classified in the Virulence and Detoxification category and about 15% in the Lipid Metabolism category. Cell wall proteins include the outer membrane proteins (OMPs) that are likely localized in the recently discovered mycobacterial outer membrane bilayer.^{79,80} By combining the use of an algorithm entirely based on physical principles to predict outer membrane proteins (OMPs) of *M. tuberculosis* with biological knowledge, Song et al.⁸¹ have predicted 144 proteins as OMPs of *M. tuberculosis*. These OMPs may participate in the uptake of hydrophobic compounds across outer membranes, efflux processes and energy-dependent uptake of nutrients. They also may

play a role in the attachment and invasion of host cell and the degradation of host's structures. Importantly, some of these OMPs would comprise the unknown outer membrane component of the inner membrane transport systems, already identified in MTBC.

The identification and characterization of cell wall and secreted proteins is critical to the understanding of bacterial survival and immune modulation in the host. In this section we describe mycobacterial cell wall proteins, including outer membrane and cell-associated secreted proteins that were demonstrated to play a role in the interaction of MTBC species with their host.

Erp. Erp (exported repetitive protein) is a cell-wall-associated surface protein, usually secreted into the culture medium with a molecular mass of 36-kDa, therefore, it is known as P36 protein⁸²⁻⁸⁴ (Fig. 2). Erp proteins have a modular organization consisting of three domains: two well-conserved N- and C-terminal domains, and a central domain containing several Pro-Gly-Leu-Thr-Ser (PGLTS) repeats which vary in number, with four repeats in *M. leprae* and 24 in *M. xenopi*. This variability of the central domain has shown to be related to virulence.⁸⁴ Although the function of Erp still remains unknown, the contribution to virulence has been well demonstrated. Berthet et al.⁸² have shown that *M. tuberculosis* and *M. bovis* BGC *erp* mutants were impaired for multiplication in cultured macrophages and in BALB/c mice compared with the wild type. The re-introduction of *erp* into the mutants restored their ability to multiply, suggesting that Erp contributes to the virulence of *M. tuberculosis*. Similarly, Bigi et al. have observed the same phenotype in an *M. bovis* *erp* mutant infecting BALB/c mice: the CFU counts in spleen and lung were markedly reduced compared with the wild-type or complemented strain.⁸⁵ Additionally, an *M. tuberculosis* *erp* mutant complemented with the *erp* from *M. leprae*, which has a reduction in the number of PGLTS repeats in the central domain, correlated well with an increase in the multiplication and damage of mycobacteria in the lung of mice.⁸⁴ This result suggests that the contribution of Erp to virulence is directly related with the extension and variability of its central PGLTS repeated domain. Besides, the Erp family is not restricted to pathogenic mycobacteria and is also present in the saprophytic ones, which suggests a more physiological role for Erp. The *erp* gene is located between *glf* and *csp*, which encode proteins involved in lipopolysaccharide biosynthesis. Given that this genomic organization is well conserved in many mycobacterial species, it is tempting to hypothesize that Erp could have a possible role in cell wall biosynthesis.⁸⁶

Fbp. Fbp (fibronectin binding protein) is a complex of three proteins: FbpA, FbpB and FbpC2, and their name derives from their capabilities to bind fibronectin (FN). This complex is commonly known as antigen 85 (Ag85) and includes Ag85A (FbpA), Ag85B (FbpB) and Ag85C (FbpC2). These proteins are encoded by the *fbpA*, *fbpB* and *fbpC2* genes, which are located in different genomic regions: *Rv3804*, *Rv1886c* and *Rv0129c*, respectively. A fourth gene *fbpD*, or *fbpC1*, related to Ag85 is annotated in the *M. tuberculosis* genome. However, FbpC1 did not show an in vitro mycolyl transferase activity, a signature of this complex.⁸⁷ The Fbp complex is the major secreted protein

constituent of mycobacterial cell culture and it is also found in association with the bacterial surface.^{78,88,89} This protein complex plays an essential role in the pathogenesis of tuberculosis. Its ability to bind FN promotes the adhesion of mycobacteria to the mucosal surface, thus facilitating its entry into the host cells.⁸⁹ However, its main contribution to the virulence of *M. tuberculosis* is likely due to its physiological role in the synthesis of cell wall lipids.

Belisle et al.³⁰ have shown that FbpA, FbpB, and FbpC have mycolyltransferase activity, required for maintaining the integrity of the mycobacterial cell envelope. All three proteins catalyze the transfer of mycolates to trehalose, leading to the formation of α , α' -trehalose monomycolate (TMM) and α , α' -trehalose dimycolate (TDM) (Fig. 2). While FbpA and FbpC2 have similar specific activities, the FbpB specific activity values decreased by up to 80% compared with FbpC2. These proteins conserve the carboxyl esterase consensus sequence (G-X-S-X-G) and have a catalytic triad formed by Ser, Asp/Glu and His, in which the Ser is the active site.³⁰ Studies on the crystal structure of FbpC2 have revealed a hydrophobic pocket and tunnel extending into the protein structure as the probable trehalose monomycolate binding site, and a large region of conserved residues in the surface of the proteins a putative site for the interaction with FN.⁹⁰

The role of Fbp proteins in the virulence of mycobacteria has been evaluated in individual mutant strains. While an *M. tuberculosis* H37Rv *fbpA* mutant exhibited a marked decrease in growth in monocyte-like human THP-1 and murine J774 macrophage cell lines compared with the parental strain, an H37Rv *fbpB* mutant grew at the same rate than the wild type in those macrophage models of infection.³¹ This could be a direct consequence of the catalytic efficiency of each protein. In an additional study, an *M. tuberculosis* Mt103 mutant in *fbpC2* gene showed 40% less mycolates in the cell wall as compared with the parental strains, although changes in the types of mycolates esterifying arabinogalactan or in the composition of non-covalently linked mycolates were not observed. Despite this profound modification in its cell wall, the *fbpC* mutant showed a similar intracellular replication and survival in mouse bone marrow macrophages as compared with the wild type.⁹¹ Altogether, these results suggest that FbpA, but not FbpB or FbpC, would be essential in the virulence of *M. tuberculosis*.

Mce. The Mce proteins are a large group of secreted or surface-exposed proteins organized in large operons. Their name Mce, mammalian cell entry, is due to the first function described for the Mce proteins: it has been shown that Mce1 conferred mycobacteria the ability to enter into mammalian cells and survive inside the macrophage.⁹² These operons comprise eight genes each and are organized in identical manner: two *yrbE* genes (A and B) followed by six *mce* genes (A, B, C, D, E and F).²² *M. tuberculosis* contains four *mce* loci: *mce1*, *mce2*, *mce3* and *mce4*, whereas *M. bovis* lacks the *mce3* locus.⁹³ Mce proteins are homologous to ATP-binding cassette transporters (ABC-transporters), presenting a typical gene arrangement of importers in which YrBEs are homologous to permeases and have a region within the penultimate cytoplasmic loop that may serve as the site of interaction to ATPases, whereas Mces are homologous to

substrate-binding proteins⁹⁴ (Fig. 2). The contribution of each Mce to virulence has been demonstrated from a set of *M. tuberculosis* knockout mutants. When *mce1*, *mce2* and *mce3* mutants were used to infect BALB/c mice by an intratracheal route, they showed an attenuated phenotype compared with the wild type.⁹⁵ The same behavior was observed when mutants for the *mce2*, *mce3* and *mce4* loci were used to infect C57BL/6 mice after aerosol infection.^{96,97} However, $\Delta mce1$ *M. tuberculosis* strain showed a hyper virulent phenotype as compared with parental or complemented strains⁹⁸ in mice infected by a systemic or intraperitoneal⁹⁵ route with this mutant. This indicates that in the absence of the Mce1 *M. tuberculosis* could trigger an active infection; therefore, a specific control of the *mce1* expression would be necessary to keep the mycobacteria proliferation in check and allow the establishment of a latent infection in the host.⁹⁸ Contrary to this, some high-throughput techniques have shown that the *mce* genes, mainly *mce1*, are required for mycobacterial survival in macrophages or mice models of infection.⁹⁹⁻¹⁰¹ Perhaps the importance of Mce proteins in MTBC virulence lies in their hypothetical role as transporters and, therefore, their absence would prevent these mycobacteria from importing essential but yet unidentified compounds. The first Mce operon with a demonstrated transport function was Mce4,⁶¹ which is involved in cholesterol import in *M. tuberculosis*. Importantly, cholesterol is essential for maintaining a chronic infection in the host as it has been described in the cholesterol metabolism section. Later on, Santangelo et al. demonstrated that the transcriptional regulator of *mce3* operon, Mce3R, not only controls the expression of the *mce3* operon but also of the *Rv1933c-Rv1935c* and *Rv1936-Rv1941* operons, which encode proteins that are predicted to be involved in lipid metabolism and redox reactions.^{102,103} Additionally, a mutant deleted in the *fadD5* gene, which is located within the *mce1* operon, shows a decreased growth in minimal medium containing mycolic acids as a carbon source.¹⁰⁴ This suggests that the Mce1 proteins also may be related to the recycling of these lipids. Furthermore, a mutant in the *mce2* operon shows an increased accumulation of sulpholipids.¹⁰⁵ Thus, although a clear role for *mce* operons has been only firmly established for *mce4*, it is reasonable in light of the results of *mce1*, *mce2* and *mce3* to postulate that these operons have evolved to fulfil specific roles, most likely related to lipid metabolism, modulating pathogenicity through changes in *M. tuberculosis* lipid pathways.

OmpATb. OmpATb is a pore-forming protein (porin) that belongs to the OmpA family of outer membrane proteins. Its β -sheet/ β -barrel structure enables it to form pores with a diameter of 1.4 and 1.8 nm, facilitating the passage of small hydrophilic molecules such as arabinose, glucose, sucrose and serine to the cytoplasm¹⁰⁶ (Fig. 2). Like other members of the OmpA family, this porin plays a role in pathogenicity. An *M. tuberculosis* mutant in the *ompATb* gene showed a significantly reduced multiplication in macrophages compared with the wild type, and its growth in lungs and spleen of BALB/c mice was markedly reduced when compared with the levels of the wild type.¹⁰⁷ The transcription of the *ompATb* gene is highly increased at low pH suggesting an activity of this porin in acidic conditions. As expected, the growth

of *ompATb* mutant at low pH is affected as well as the uptake of some water-soluble metabolites such as serine.¹⁰⁷ These observations suggest that OmpATb is a pore-forming protein that functions under acidic conditions. Therefore, in the environment prevailing within the phagosome, OmpATb allows the bacteria to continue acquiring molecules and, therefore, makes its survival possible.

HbhA. HbhA (heparin-binding hemagglutinin) is the major adhesin exposed at the surface of the cell (Fig. 2). This protein binds sulphated glycoconjugates like heparin, promoting the attachment of the mycobacteria to the epithelial cells and fibroblasts, but not to macrophage-like cells. In addition, the protein promotes the agglutination of rabbit erythrocytes and also induces mycobacterial aggregation,¹⁰⁸ which gives the bacteria the ability to form a primary biofilm. HbhA is a 28-kDa protein with three functional domains: a large N-terminal domain of 81 amino acids consistent with an α -helical coiled-coil region, which promotes bacteria-bacteria interaction, a trans-membrane domain of 18 amino acids residing near the N-terminus, which is the anchoring to the cell wall, and a C-terminal domain with a Lys-Pro-Ala-rich repeat that mediates binding to proteoglycans.^{109,110} In addition, the relevance of HbhA in virulence has also been demonstrated in an in vivo model of infection. The disruption of the *M. tuberculosis* M103 or *M. bovis* BCG *hbhA* gene markedly affects mycobacterial interactions with A549 pneumocytes but not with murine J774 macrophage cells. Also, when the mutants are inoculated into BALB/c mice, they are severely impaired in spleen colonization, but not in lung colonization compared with the parental or complemented strains. These results indicate that HbhA is required for extrapulmonary dissemination, and that interactions with non-phagocytic cells have an important role in the pathogenesis of tuberculosis.¹¹¹ Interestingly, HbhA is immunogenic in humans during mycobacterial infections¹⁰⁸; additionally, coating wild-type mycobacteria with anti-HbhA antibodies also impaired dissemination in a mouse model of infection; which suggests that the antibody responses to HbhA may be playing an important role to immune protection against tuberculosis.¹¹¹

PstA1 and PhoT. PstA1 and PhoT (encoded by *pstA1* and *phoT*, respectively) are proteins involved in the transport of inorganic phosphate. Whereas *pstA1* is clustered together with *pstC2* and *phoS2* (also called *pstS3*) in the same operon, *phoT* is located elsewhere in the genome. PhoS2 is the substrate-binding protein, PstC2 and PstA1 are the permeases containing the membrane spanning domain (MSD) and PhoT has homology to the nucleotide-binding domain protein, responsible for the energy coupling to the transport system^{99,112} (Fig. 2). *M. tuberculosis* contains three ABC phosphate transporters: PstS-1 (described below with the 38-kDa protein), PstS-2 and PstS-3. All of them are involved in phosphate import during starvation, a condition prevailing inside the phagosome. Rengarajan et al.⁹⁹ showed that the PstS-3 transporter plays a role in virulence, although Sassetti and Rubin had previously shown that it was not essential for mycobacterial survival in mice.¹⁰⁰ The *M. tuberculosis* transposon mutants in the *pstA1* or in the *phoT* genes were more sensitive to grow in a phosphate-limited culture compared with the wild type;

however, they grew at the same levels in a high phosphate concentration cultures.⁹⁹ Mutants grew poorly in resting and activated macrophages compared with the wild type, although the attenuated phenotype could be partially complemented by overexpression of each wild-type gene. In addition, the *phoT* mutant has shown a reduced growth in mouse lungs but not in spleen or liver,⁹⁹ compared with the wild-type or complemented strains. These results clearly indicate that PstS-3 is required for phosphate uptake and survival within macrophages.⁹⁹

CaeA. CaeA, also known as Rv2224c, is a carboxylesterase located in the cell surface. The protein is an esterase/lipase, preferentially hydrolyzing ester bonds of substrates with intermediate carbon chain length, about 3 to 7 carbon atoms.¹¹³ The enzyme is a 54-kDa monomer with the active-site consensus sequences (G-X-S-X-G), where the replacement of Ser²¹⁵, Asp⁴⁵³ and His⁴⁷⁷ by Ala completely abolishes the esterase activity, suggesting that these residues form the catalytic triad with Ser²¹⁵ as the active site residue. Its serine active site is characteristic of α/β hydrolase-fold family members, namely proteases, esterases, and lipases. In a former study it has been shown that CaeA is required for full virulence of *M. tuberculosis* in mice.¹¹³ A deletion mutant has been used to infect BALB/c mouse, showing reduced CFU load in lungs and spleen and diminished lung pathology as compared with the wild-type or to complemented strains.¹¹³ Similar results have been reported with an *M. tuberculosis* Rv2224c insertional mutant when used to infect C57BL/6 8 mice.¹¹⁴ In addition, these authors have shown that this mutant is hypersusceptible to acid or oxidative stress (see section on "Proteins Inhibiting Antimicrobial Responses of the Macrophage"). Lately, Rengarajan et al. also demonstrated that Rv2224c is critical for *M. tuberculosis* virulence in vivo. In C57BL/6 infected mice, the mutant survived significantly longer and caused reduced lung pathology than the wild type. In primary macrophages at early stages of infection, both wild-type and mutant strains grew equally well, but the mutant failed to continue growing, probably because of its inability to modulate the innate immune control of infection. Interestingly, these authors have reported that Rv2224c is needed to the release of GroEL2 from the cell wall of *M. tuberculosis*, inferring a protease activity for this protein. GroEL2 is a highly expressed, immunodominant stress-induced protein that is present as both a full-length and smaller N-terminally processed form.¹¹⁵ The role of Rv2224c in the virulence of *M. tuberculosis* is unquestionably, possibly due to its role as lipase¹¹³. Although it has been observed that some proteases may also have a lipase activity, whether or not GroEL2 is a direct substrate for Rv2224c, remains unclear and awaits further biochemical characterization.

KefB. The potassium efflux system KefB functions via potassium/proton antiport. Bacteria inside phagosomes can release potassium from their cytoplasm via KefB and uptake proton from the phagosomal lumen, increasing luminal pH. This latter interferes with the phagosomal maturation and hence with the elimination of the bacteria. An *M. tuberculosis* *kefB* mutant generated by transposon insertion localized mostly in acidified phagosomes,¹⁰¹ suggesting that the role of KefB is to avoid the phagosomal acidification induced in macrophages in response to bacterial infection. However, paradoxically, the opposite has been

observed in an *M. bovis* BCG mutant in the *kefB* gene (BCG Δ *kef*). The BCG Δ *kef* mutant exhibits an increased intracellular survival phenotype in resting and activated murine J774 macrophages compared with the wild-type BCG Pasteur.¹¹⁶ Despite the absence of KefB, this mutant retains the capability to inhibit phagosome acidification. In addition, the mutant is able to inhibit the induction of antimicrobial mechanisms from the macrophages, such as ROS. The production of ROS by macrophages infected with BCG Δ *kef* decreased in relation with those infected with wild-type BCG, suggesting that reduction of the macrophage oxidative burst could be the cause of the increased intracellular survival of BCG Δ *kef*.¹¹⁶ Therefore, the production of ROS in the mycobacterial phagosome, paradoxically, would depend on the bacterial potassium transporter KefB, whose activity is altering the ionic contents of the phagosome and promoting the production of ROS. So, the role of KefB in the virulence of *M. tuberculosis* is still controversial and requires further studies.

Opp-Dpp ABC transporter. Two predicted operons encoding permeases involved in the uptake of small peptides are annotated in the *M. tuberculosis* H37Rv genome based on homology to other described transporters: OppABCD (oligopeptide permease, Rv1283c-Rv1280c) and DppABCD (dipeptide permease, Rv3666c-Rv3663c). Different approaches have been used to elucidate their function and role in bacterial survival. Based on the results from Green et al.¹¹⁷ and Flores-Valdez et al.,¹¹⁸ it is now clear that the Rv3666c-Rv3663c locus encodes an oligopeptide transport system, whereas the Rv1283c-Rv1280c locus encodes a dipeptide system. As a consequence, special attention should be paid to the nomenclature in bibliography. Two insertional mutants in the ATP-binding component of both operons (which renders the transporter not functional because the ATP hydrolysis is impaired) have shown different results. BCG Rv1281c (formerly *oppD*) transposon-insertion mutant in inactivated murine macrophages failed to show an affected survival,¹¹⁷ whereas an Rv3663c (formerly *dppD*) mutant strain, generated by allelic exchange in H37Rv, showed an affected survival at the initial phase of infection in mice.¹⁰⁰ Along the same line of evidence, in a microarray-based screening of a BCG transposon library in macrophage culture, *dppC* disruption has shown among the most attenuating mutations of that study.¹⁰¹ Moreover, Flores-Valdez et al.¹¹⁸ obtained an *opp* (Rv3666c-Rv3662c) *M. tuberculosis* knockout strain and assessed its virulence in BALB/c mice. This *opp* mutant did not show differential virulence phenotype in mice during the active phase of infection. However, bacterial load in lungs and spleen in the chronic phase of infection was diminished when compared with the wild-type strain. In addition, the survival time of the *opp* mutant in infected animals was higher than in the wild type, but the reintroduction of a copy of *opp* into the mutant strain failed to restore the wild-type phenotype. These authors have proposed that Opp incorporates oligopeptides that modulate intracellular signaling pathways, favoring the survival of *M. tuberculosis* inside cells. Indeed, Opp was required to modulate the expression of several genes like *fasI*, *desA3*, *icl*, *fadE13* and *PE13* among others, most of them encoding surface-exposed molecules such as mycolic acids, PDIMs as well as PE-family proteins. An

interesting hypothesis is that these permeases are involved in the import of peptides or lipopeptides to signal the need to adequate/remodel cell wall envelope components. A comparable system is the basis of the natural uptake of DNA (competence) displayed by *Bacillus subtilis*, which uses formation and import of oligopeptides to signal metabolic stages. Regarding the important role of lipid molecules in the mycobacterial physiology and in the molecular mechanisms of pathogenesis displayed by *M. tuberculosis*, more studies are undoubtedly warranted.

CtaC. CtaC is the subunit II of the cytochrome *c* oxidase important for growth under aerobic conditions. An in silico analysis showed the cytochrome oxidase domain is on the extracytoplasmic face of the membrane.¹¹⁹ CtaC has been predicted to be essential in *M. tuberculosis* H37Rv. An *ctaC*::Tn_{bla}TEM-1 mutant, which expresses a version of CtaC truncated in the final 13 amino acids of the C-terminus, was able to grow in vitro, but showed a rough and spread out colony phenotype compared with the wild-type strain. This mutant displayed a growth defect in macrophages, suggesting that CtaC has a role in virulence. However, no experiment has been done to rule out the possibility that the attenuated phenotype of the *ctaC* mutant was due to a polar effect on the downstream *ctaF* gene, which has been predicted to encode for an additional subunit of the cytochrome *c* protein.¹¹⁹

Lipoproteins. Lipoproteins (Lpps) constitute a major component of the cell envelope in mycobacteria. Bioinformatics studies have predicted that the *M. tuberculosis* genome potentially encodes 48 to 99 lipoproteins (depending on the different algorithms used) representing 1.2 to 2.5% of the proteome.^{120,121} However, only a few experimental reports have found evidence of acylation. Lipoproteins in MTBC are predicted to be associated to diverse cellular functions, including transport, cell wall metabolism, cell adhesion, signaling and protein degradation and thus some lipoproteins will play a significant role in virulence. The association of Lpps to virulence was clearly demonstrated in 2004. The recent discovery of novel cell envelope associated potential Lpps through Triton X114 phase separation coupled to mass spectrometry¹²² emphasizes that there is much to be explored in this protein family. Different evidence indicates that Lpps could be directly or indirectly involved in virulence and hence data from recent research are integrated herein this section.

The first gene to be described in this section is *LspA*, the lipoprotein signal peptidase responsible for removal of the signal peptide following trans-acetylation and translocation. The peptidase cleavage generates the mature form of the lipoprotein that is anchored in the membrane.¹²¹ Sander et al. disrupted *lspA* by allelic replacement in *M. tuberculosis*. The obtained *lspA* mutant, impaired in lipoprotein synthesis, resulted markedly attenuated in the relatively resistant mouse strain BALB/c and the more susceptible strain CBA/J to infection with *M. tuberculosis*.¹²³ Early in the 1990s, Young and Garbe had identified four lipoprotein antigens in *M. tuberculosis* through detergent phase separation and metabolic labeling.¹²⁴ Since then, others Lpps have been identified or predicted in silico.^{120,121}

LppX. LppX was primary described as a mycobacterial protein antigen (22-kDa) common to *M. leprae* and the MTBC.¹²⁵ The

protein is secreted and is also highly expressed in the bacterial cell wall and membrane compartment but has not been found in the cytosol.¹²⁶ STM strategy has established the link of this Lpp to virulence. The disruption of *lppX* had no effect on in vitro growth, but the mutant obtained by the disruption of this gene showed an attenuated phenotype in BALB/c mice.¹²⁷ This result was concordant with that obtained in a previous study in C57BL/6J mice.¹⁰⁰ Chromosomal localization of *lppX* in a region of genes involved in PDIM metabolism has led to a functional characterization of LppX related to this complex lipid. A biochemical approach through lipid fractionation coupled to MALDI-TOF has shown that the wild type and its isogenic *lppX* mutant produced the same types and amount of PDIM molecules. However, while 36% of the PDIM synthesized by the wild type was found in the culture filtrate, no PDIM was detected in the culture filtrate of the mutant; it remained associated to the cell wall and cytosol plus plasma membrane fractions. In addition to these results, a structural biology study has provided strong support regarding a possible role of this protein as a PDIM carrier transport from the outlet leaflet of the plasma membrane to the outer membrane.¹²⁷ In this way, the possible contribution to virulence could be explained through the role of PDIM as explained above.

LpqH (19-kDa lipoprotein antigen). LpqH has been first described as a 19-kDa lipoprotein antigen of *M. tuberculosis*.¹²⁴ A recent study has described this Lpp as a putative glycoprotein.¹²⁸ The *M. tuberculosis* 19-kDa glycolipoprotein can inhibit MHC-II antigen processing and presentation in macrophages.¹²⁹ This inhibition occurs by blocking gamma interferon (IFN- γ) signaling through a Toll-like receptor-2 dependent (TLR-2) mechanism. The 19-kDa lipoprotein, as well as synthetic lipopeptides, induces dendritic cell (DC) maturation. The lipid moiety of the lipopeptide has been found to be essential for induction of DC maturation.¹³⁰ In addition to its powerful immunomodulatory properties, exposure of neutrophils to the *M. tuberculosis* 19-kDa lipoprotein promotes neutrophil priming and activation.¹³¹ Exposure of macrophages to this protein may also induce apoptosis, allowing escape and dissemination of the bacilli.¹³² Recently, it has been shown that the 19-kDa lipoprotein activates autophagy in human monocytes and, thus, could exert antimycobacterial activity through the induction of this mechanism.¹³³ Besides, the 19-kDa Ag of *M. tuberculosis* has been described as a major adhesin that binds the mannose receptor of THP-1 monocytic cells and promotes phagocytosis of mycobacteria.¹³⁴ These properties reinforce the pleiotropic effects of this molecule at different levels of the infection process. Even more contradictory results have been published: for instance, while a report has shown that despite inducing an Ag-specific Th1 response, overexpression or addition of the 19-kDa Ag has a detrimental effect on protective efficacy of different mycobacterial vaccines such as *M. vaccae* or BCG.^{28,135} Other authors, however, have reported that neither overexpression of the 19-kDa Ag, nor deletion of the endogenous 19-kDa Lpp gene alter the ability of BCG to protect against *M. tuberculosis* challenge in a mouse model.¹³⁶ Interestingly, *M. tuberculosis* 12646 and SI strains that do not express the 19-kDa Lpp in vitro and in vivo, exhibit lower

virulence than the transformant-producing native 19-kDa glycolipoprotein, as assessed by bacterial loads and lesions in infected organs.¹³⁷ Later on, a high-throughput mutagenesis screen showed that disruption of the 19-kDa Lpp gene is associated with moderately reduced bacterial load in mice.¹⁰⁰ More recently, an *M. tuberculosis* H37Rv Δ lpqH mutant displayed high attenuation in C57BL/6 mice; a phenotype that was lost upon complementation of the mutant.¹³⁸

LprG (27-kDa lipoprotein Ag, P27). LprG is a secreted surface-expressed lipoprotein antigen, which together with 19-kDa Lpp Ag is one of the most extensively studied lipoproteins (Fig. 2). It has been shown that it is also a glycoprotein¹²⁸ and described as an important antigen of *M. bovis* and *M. tuberculosis*^{139,140} but not exclusive of the MTBC. In spite of the Th1 immune response in BALB/c mice induced by this antigen, an adverse effect on the protection afforded by BCG has been observed.¹⁴⁰ Moreover, co-administration of LprG with *M. tuberculosis* aggravates the infection,¹⁴¹ suggesting that the 27-kDa Lpp plays a role in *M. tuberculosis* infection by inducing increased suppression of the immune response. The gene that encodes LprG constitutes an operon together with *Rv1410c* gene,¹⁴² usually known as *p55*. The *p27-p55* (*Rv1411c-Rv1410c*) operon, also called *lprG-p55*, is a bicistronic operon conserved across several non-pathogenic and pathogenic mycobacterial species.¹⁴³ It has been suggested that *Rv1410c* codes for the P55 protein, an antibiotic efflux pump, since overexpression of *M. tuberculosis* P55 in *M. smegmatis* confers resistance to streptomycin and aminoglycosides.¹⁴³ However, a later study in which *M. tuberculosis* P55 was overexpressed in *M. smegmatis* failed to demonstrate this phenotype.¹⁴⁴ In spite of this, evidence suggests that P55 clearly functions as a transporter. This is also supported by the homology of P55 to other major facilitator superfamily pumps and because it is a target for MDR (multidrug resistance) inhibitors such as reserpine and verapamil.^{143,144} Sequence homology between LprG and LppX may also suggest a common function, as mentioned above for LppX, related to lipid transport. Several studies have shown that cell wall composition is altered in mycobacterium mutants defective on *lprG* and *p55*.^{144,145} Thus, LprG and P55 are required for the translocation of cell wall components or their precursors. Knockout of *lprG* or its operon resulted in attenuated growth and survival in mice and macrophages.^{100,146,147}

In addition, two studies have provided evidence of a cooperative effect between LprG and P55. In the first study, the altered phenotypes of an *M. smegmatis* strain lacking both *p55* and *lprG* could be complemented by a plasmid encoding both genes, but none of the genes alone could restore the phenotype.¹⁴⁴ Moreover, the second study has shown that complementation of *M. bovis* mutant (lacking the operon) with either *lprG* or *p55* failed to fully complement the wild-type phenotype.¹⁴⁶ These studies suggest the importance of both components on virulence.

Recently, it has been proposed that LprG serves as a carrier to facilitate assembly or trafficking of glycolipids to the mycobacterial cell wall, in this way contributing to virulence.¹⁴⁸ Additionally, LprG has been recognized as a ligand for the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor in *M. bovis* BCG. DC-SIGN has an important

role in mediating adherence of mycobacteria species, including *M. tuberculosis* and *M. bovis* BCG, to human dendritic cells and macrophages. DC-SIGN is a C-type lectin receptor, and interactions with mycobacterial cells are believed to occur via mannosylated structures on the mycobacterial surface.¹⁴⁹ This study provides new insights on the role of LprG on host-pathogen adhesion. The mechanisms through which this gene/operon could play a key role on virulence may be explained by its contribution to *M. tuberculosis* cell wall assembly and/or by its contribution to the delivery of glycolipids to TLR2.

PstS-1 (38-kDa Ag; *phoS* or *phoS1*). PstS-1 belongs to the ABC phosphate transporters family (Fig. 2), homologous to the Pst system in *E. coli*. PstS-1, the phosphate binding subunit, is encoded by a gene surrounded by *pstB*, *pstC-1* and *pstA-2* within a potential operon (*pstB*, *pstS-1*, *pstC-1* and *pstA-2*).¹⁵⁰ This protein has been described as one of the first glycoproteins reported in *M. tuberculosis*.¹⁵¹ It has also been described as an immunodominant antigen in TB patients.¹⁵² Later on, through detergent phase separation and metabolic labeling, it was classified as a glycolipoprotein.¹²⁴ It has been shown that this 38-kDa glycolipoprotein possesses structural motifs to interact with both TLR2 and Toll-like receptor 4 (TLR4). In addition, the intact protein moieties of the 38-kDa Ag would be responsible for the induction of the pro-inflammatory cytokines, TNF- α and IL-6 induction in human primary monocytes. Purified 38-kDa glycolipoprotein induced the activation of ERK1/2 and p38 MAPK via TLR2 and TLR4, and the subsequent TNF- α and IL-6 in human primary monocytes. This line of evidence suggests that this antigen could play a role in early inflammatory responses during mycobacterial infection.¹⁵³

Further investigation has been focused on this antigen as evidence indicated its possible role in phosphate metabolism.¹⁵⁴ In immunolocalization experiments, gold-particles labeling 38-kDa Ag were located mainly in the wall and on the outer surface of the bacilli. This location is in agreement with the possible role of P38 in phosphate metabolism. It is also consistent with previous studies suggesting that P38 is a secretion antigen.¹⁵⁵ Additionally, this protein is overexpressed under phosphate starvation.^{155,156} *pstS1* and *pstS2* knockout strains showed a significantly reduced multiplication rate within mouse peritoneal macrophages and were attenuated in an in vivo infection model.¹⁵⁷ This study has also provided important evidence on a possible role of Pst-1 and Pst-2 in phosphate uptake from media with low phosphate concentration contributing to the intracellular survival of *M. tuberculosis*.

LpqY. LpqY is a component of a putative sugar ABC transport of *M. tuberculosis* and *lpqY* forms an operon with *sugC*, *sugB* and *sugA*. Different studies based on TraSH have identified *lpqY* as a gene required for growth in mice and in macrophages.^{99,100} This could mean that sugars (not only host lipids) could be important for infection. A study based on this ABC transporter has provided new insights into the role of sugar transporters in pathogenesis. It has been shown that this importer plays a role in recycling of extracellular trehalose, released from trehalose-containing molecules synthesized by the bacillus.¹⁵⁸ SCID mice infected via the aerosol route with the Δ lpqY or Δ sugC mutants survived

significantly longer than mice infected with the wild-type or the complemented mutant strains. In C57BL/6 mice, trehalose transporter mutants showed a severe growth defect in lungs of infected animals during the acute phase of infection, but failed to persist during the chronic phase, albeit at a strongly reduced bacillary organ burden.¹⁵⁸ Genetic complementation of the mutants fully restored virulence in this model. This finding highlights the crucial importance of the LpqY-SugA-SugB-SugC transporter-mediated uptake of trehalose in the virulence of *M. tuberculosis*.

ModA. ModA (molybdate-binding lipoprotein) is encoded in the *modABC*-operon of the ABC-type transport system for molybdenum.²² Further characterization of the attenuated mutants from a STM library screened in mice has shown that the interruption of this putative gene confers moderate attenuation.⁴² This result has implicated molybdenum uptake in virulence. In the majority of molybdenum-containing enzymes, the metal is coordinated to the dithiolene group of molybdopterin (MPT) to form molybdenum cofactor (MoCo), with the exception of nitrogenases, in which molybdenum is coordinated in an iron-molybdenum cofactor.¹⁵⁹ Deficient transport of molybdenum may, therefore, impact MoCo biosynthesis and lead to a function deficiency of several molybdenum-dependent enzymes essential for the survival of the bacteria.¹⁵⁹ However, involvement of these enzymes in virulence might be further investigated.

Other Lpps. Wide genome screening of *M. tuberculosis* H37Rv mutants has allowed the identification of additional Lpps encoding genes specifically required for mycobacterial survival *in vivo*¹⁰⁰ or *in vitro*.⁹⁹ Several Lpps have been identified in both studies: *lpqT*, *lpqY*, *lprG* (described elsewhere), *lpqZ* and *lprK*. On the other hand, *lppX* (described elsewhere) and *lprN* are exclusively required for *in vivo* survival,¹⁰⁰ whereas *lppP* and *lprO* have been predicted to be required for growth in macrophages.⁹⁹ Additional studies are needed to fully characterize the function of these Lpps and their implication in virulence.

The cell envelope represents an interphase considered relevant in the host-pathogen interaction and several studies have been performed to reveal potential drug targets and vaccine candidates. Knowledge of cell wall proteins and other proteins, together with their biological function, are critical to understand how mycobacteria survives and modulates the host immune response. The main virulence factors described in this section are proteins involved in transport of metabolites (hydrophilic molecules, inorganic phosphate, peptides or lipids) or in lipid and glycolipid synthesis. In relation to the latter function, the degradation and reconstruction of the cell wall keep its architecture intact which is necessary for virulence and survival of mycobacteria in the host. Several lines of evidence point out to the Ag85 complex as a remarkable multitask protein component, with adhesive properties and related to process of cell wall synthesis. As mentioned above, the crystal structure of Ag85C has revealed several potential targets to design preventive or therapeutic strategies as the location of the trehalose monomycolate binding site and the region for fibronectin interaction.⁹⁰ Additionally, several novel tuberculosis vaccines currently in

clinical trials involve the use of these antigens: Ag85B fused to other antigens^{160,161} or Ag85A expressed on modified vaccinia ankara virus.¹⁶² Nowadays, this Ag complex remains as a good candidate to control the infection and attention on its use has not declined.

Lpps are part of the uncommon cell envelope of mycobacteria, and its acylation allows them to anchor and/or sort to the cell surface. Their main function is structural but they may also be involved in synthesis and/or transport of important components of the cell envelope. Besides its clearly structural role, Lpps could act as stimulatory and inhibitory ligands of TLRs allowing modulation of the immune response to mycobacteria. This has been addressed and confirmed by targeting of these genes through mutagenesis experiments used to reveal their relevance in host-pathogen interaction. The export of a variety of potential virulence factors through membrane vesicles (MV)^{163,164} could be considered a process directly involved with the cell envelope. This mechanism has also been described in mycobacteria and can serve to deliver virulence factors to other compartments of the eukaryotic cell or to their incorporation into host cell-derived exosomes.^{164,165} Despite that several different molecules are present in *M. tuberculosis* complex-MVs, Lpps—such as 19-kDa Lpp, LprG, and LppX—constitute a large fraction of them (20% of total proteins), underlying their importance in the interplay with the host immune system.¹⁶⁵ A detrimental effect of MVs for the host during *M. tuberculosis* infection has been also observed, since mice pretreated with pathogenic-MVs developed a more acute inflammation and higher bacterial loads in the lungs compared with control and nonpathogenic MVs-treated mice. Moreover, in the same study, preexposure to MVs also increased dissemination of bacilli from the lung to other lymphoid organs.¹⁶⁵

These observations highlight the intriguing mechanisms involved in immune response modulation, in which Lpps appears as relevant players. Most of the Lpps characterized as virulence factors by mutagenesis have also been described as antigenic, some of them with potential diagnostic value. Surprisingly, no deletion of the MPT83 antigen (or its counterpart, MPB83 in *M. bovis*), also described as a glycolipoprotein,¹⁶⁶ has been reported yet; however, it has been successfully evaluated as part of a DNA and protein vaccine in a mice model of tuberculosis.^{167,168}

Secretion systems. Protein secretion is a very important mechanism in bacterial functioning and operation in their natural surrounding environment for adaptation and survival. Moreover, these systems are also essential for interacting with host cells by exporting toxins/signal proteins that allow different bacterial species to cause pathology. Even more, secretion systems have evolved, duplicating and diverging to carry on specific functions; some of them are involved in pathogenicity, leading to the description of a large number of such systems. Several gene clusters encoding proteins that are secreted into the environment via specific pathways have been identified in mycobacteria; therefore, they are considered important for mycobacterial pathogenesis.

Recent evidence shows that mycobacteria have developed a novel and specialized secretion system for the transport of extracellular proteins across their hydrophobic and highly

impermeable, cell wall. Abdallah et al. have discussed this novel secretion pathway and considered variants that are present in various Gram-positive bacteria. Since the composition of this secretion system is unique and of general importance, these authors have proposed that, in line with the accepted nomenclature, it should be called type seven secretion system (T7SS).¹⁶⁹ *M. tuberculosis* contains a total of five T7SS, also called ESX, that show similarity in gene content and gene order. These systems contain genes conserved in four of the five systems named as ESX conserved components (Ecc) and also genes coding for proteins defined as ESX-1 secretion-associated proteins (Esp). Strikingly, at least four types of secretion systems are encoded in the *M. tuberculosis* H37Rv genome, but only secretion systems type II and VII are involved in virulence.

Two members of T7SS, ESX-1 and ESX-5, have been shown to be involved in virulence. Both affect the cell-to-cell migration of pathogenic mycobacteria.

The ESX-1 secretion system. A 9.5 kb fragment absent in BCG encodes for several genes of the T7SS in bacteria. ESAT6 (6-kDa early secretory antigenic target, also known as ESXA) and CFP10 (10-kDa culture filtrate protein, also known as ESXB) are two relevant proteins associated to virulence in MTBC that are secreted by T7SS. The specialized T7SS for ESXA and ESXB is called ESAT6 secretion system 1 (ESX-1). ESAT6 and CFP10 are

needed for full virulence of MTBC species.¹⁷⁰ Importantly, the genes coding for T7SS are absent in *M. microti* and in the BCG vaccine. ESAT6 and CFP10 genes are located in a segment called Region of Difference 1 (RD1).¹⁷¹ **Figure 3A** shows the genomic region where ESX-1 is placed.

ESAT6 and CFP10 are small proteins, around 9 and 10 kDa respectively, with very high self-affinity and appear as a heterodimer in culture supernatants. Both proteins are part of a large protein family in mycobacteria and some Gram-positive bacteria. This family is characterized by a WXG signature located in the central part of the around 100 aa size protein.¹⁷² ESAT6 and CFP10 have been described as dominant antigens recognized by T-cells in natural infection in humans and bovines and in experimentally infected animals.^{173,174}

By means of genetic manipulations, the segment corresponding to RD1 have been deleted in wild-type *M. tuberculosis*¹⁷¹ or reinserted in BCG. The *M. tuberculosis* mutant in RD1 was attenuated in comparison to its parental strain but more virulent than BCG,¹⁷⁵ which has other genomic regions deleted (in addition to RD1). This RD1 *M. tuberculosis* mutant has been useful for complementation studies that allowed the identification of genes in *ESX-1* locus required for the full virulence of *M. tuberculosis*. The BCG strain complemented with RD1 gained virulence, measured as persistence and growth in mice, and secretion of ESAT6 and CFP10.¹⁷¹

The different nomenclatures of ESX-1 are shown in **Table 2**. *ESX-1* is conserved in many other mycobacteria. For instance, in *M. smegmatis* *ESX-1* encodes for genes involved in transfer of genetic material by conjugation. It is also present in *M. leprae* but absent in *M. avium* complex species. In the complemented BCG, it has been observed that only the use of a full RD1 DNA fragment, contrasting to smaller fragments, restored secretion of ESAT6 and CFP10 and increased virulence. This was the first indication that RD1 encodes for a secretion system.

M. tuberculosis uses the ESX-1 secretion system to deliver virulence proteins during infection of host cells (**Fig. 3B**). Furthermore, the finding that the C-terminal signal sequence of CFP10 is sufficient to secrete yeast fusion proteins via the ESX-1 system supports that CFP10 is a secreted protein.¹⁷⁷ Sequence analysis of the ESX-1 proteins shows that EccA has an ATPase signature. The proteins EccB, EccCa and EccD exhibit 1, 3 or 11 predicted transmembrane domains, respectively, and they probably are the secretion apparatus. CFP10 has a C-terminal signal sequence, recognized by EccCb, that itself interacts with the membrane protein EccCa. A point mutation in this signal sequence abolished the binding of CFP10 to EccCb and prevented secretion of ESAT6 and CFP10.¹⁷⁷ *Mycobacterium marinum* has a T7SS very similar to that of *M. tuberculosis*, and its polar localization in this bacillus has been demonstrated by confocal and electronic microscopy.¹⁷⁸ Similar results have been obtained in *M. smegmatis*.¹⁷⁹

EspB is secreted through ESX-1 and, after its secretion, is cleaved yielding a 50-kDa protein from the original 61-kDa. The EspB C-terminus is dispensable for its own secretion, as the expression of a truncated form of EspB in an *espB* transposon mutant led to a normal secretion of EspB. In *M. marinum*,

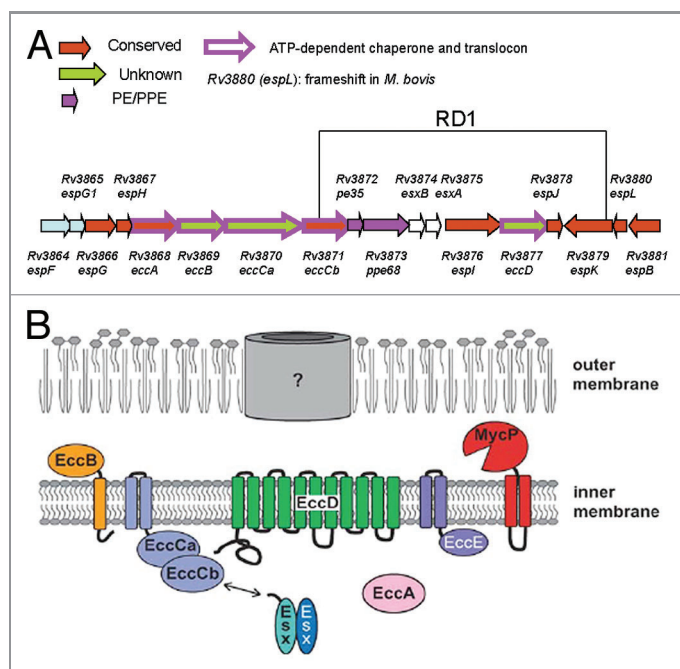


Figure 3. Schematic representation of the ESX-1 secretion system. (A) Schematic organization of the *M. tuberculosis* genomic region containing the RD1 genes. (B) Model. The abbreviation ecc stands for esx conserved component, whereas esp stands for ESX-1 secretion-associated proteins. The topology of the different proteins in the cytoplasmic membrane is shown and refers to the ESX-1 cluster based on predictions made using the MEMSAT3 algorithm. Note that the channel drawn in the outer membrane of this model refers to a hypothetical pore, whose existence has not been experimentally demonstrated. Reproduced from Bitter et al.¹⁷⁶

Table 2. Nomenclature of proteins involved in the ESX-1 type seven secretion system in *M. tuberculosis*¹⁷⁶

Rv number	Other usual name	New nomenclature
Rv3614	Snm 10	EspA
Rv3615	Snm 9	EspC
Rv3616		EspD
Rv3849		EspR
Rv3864		EspF
Rv3865		EspG1
Rv3866	Snm 5	EspG
Rv3867		EspH
Rv3868		EccA
Rv3869	Snm 6	EccB
Rv3870		EccCa
Rv3871		EccCb
Rv3872	PE-35	PE-35
Rv3873	PPE-68	PPE-68
Rv3874	CFP10	ESXB
Rv3875	ESAT6	ESXA
Rv3876	Snm 3	EspI
Rv3877	Snm 4	EccCd
Rv3878	Tb-27	EspJ
Rv3879		EspK
Rv3880		EspL
Rv3881		EspB
Rv3882		EccE
Rv3883	MycP1, Snm 8	

however, the C-terminus is essential for the interaction of EspB with ESAT6, maintenance of intracellular levels of ESAT6 and secretion of ESAT6 and CFP10, indicating that cleavage of EspB could have a regulatory function in ESX-1 secretion.¹⁸⁰

In addition to ESAT6, CFP10 and EspB, three other substrates of the ESX-1 system have been described: EspA, EspB, EspC and EspR.¹⁸⁰⁻¹⁸⁵ An unusual feature that distinguishes ESX-1 from other systems is that the secretion of all substrates is mutually dependent. For example, secretion of EspA is blocked in an ESAT6 mutant, and vice versa.¹⁸¹

In turn, EspF and EspG1 are virulence factors that are not part of the ESX-1 system, because their disruption did not impact the secretion and T cell recognition of ESAT6/CFP10 but still caused severe attenuation in BMDM, although no attenuation was observed in another cell types, such as pneumocytes.

In aerosol-infected C57BL/6 mice, 3- and 4-log reductions in bacillary load were observed in lungs for *espG1* and *espF* mutants, respectively.¹⁸⁶

The mode of action of ESAT6 in eukaryotic cells has not been precisely defined. The most frequently reported observations are the lysis of cells and/or membranes. Guinn et al. have reported that ESAT6 deletion mutants of *M. tuberculosis* are capable to multiply within THP-1 macrophage cells lines but fail to spread

to uninfected macrophages. Moreover, ESAT6 destabilizes and lyses liposomes, whereas CFP10 lacks these characteristics.¹⁸⁷ Thus, individual RD1-region genes are required for export of ESAT6/CFP10 and for virulence of *M. tuberculosis*.

Moreover, the fact that certain single amino acid changes in ESAT6 fail to prevent secretion of the modified ESAT6 molecules, but cause attenuation of the recombinant *M. tuberculosis* strains argues in favor of ESAT6 being a secreted effector molecule.

It has been recently described that translocation of *M. tuberculosis* from the phagosome into the host cell cytoplasm at later stages of infection is facilitated by ESAT6 and CFP10.¹⁸⁸ While at days four to seven after infection with *M. tuberculosis* more than one-third of the non-apoptotic human dendritic cells contained translocated bacteria, this effect was not observed when BCG or *M. tuberculosis ESX-1* transposon mutants were used for infection.¹⁸⁸ These observations suggest that the ESX-1 system might provide *M. tuberculosis* with the required tools for escaping from the phagosomal compartment of professional phagocytic cells and/or releasing ESAT6 proteins to the cytoplasm, where they gain access to the class I-processing machinery contained in the proteasome. Such events would explain the recruitment and activation of CD8⁺ T cells found in the lungs of mice aerosol infected with *M. tuberculosis*. These are strong indications that ESX-1 secreted proteins reach the eukaryotic cytoplasm. In addition, Pathak et al. have demonstrated that ESAT6 prevents antigen-presenting cell function by inhibition of TLR signaling pathways, which in turn reduces IL-12 production by THP1 macrophages and inhibits macrophage apoptosis signals as well.¹⁸⁹

A transcription factor named EspR, encoded by *Rv3849*, binds to the *espACD* operon promoter and is then secreted from *M. tuberculosis* by the ESX-1 system.¹⁸⁵ This operon is under positive regulation control of EspR but its activation is turned off when EspR is secreted via the ESX-1 system, leading to downregulation of *espACD* transcription.

Transcriptomic analyses of *M. tuberculosis* have also shown that PhoP regulates the expression of the *espACD* operon, which are significantly less expressed in the *phoP* inactivated strain.¹⁹⁰ This and other results remark the influence of PhoP-PhoR regulation on ESX-1 activity.

In addition, serine protease MycP1 post-transcriptionally regulates the ESX-1 secretion activity, being required for substrate secretion through ESX-1 as demonstrated by the deletion of *mycP-1*¹⁹¹. The mutagenic inactivation of MycP1 proteolytic activity increases secretion of ESX-1 substrates and attenuates the virulence of *M. tuberculosis* in chronic infection models¹⁹¹ (see section on “Serine proteases”). Therefore, MycP1 contributes to the fine-tuning of ESAT6 and CFP10 secretion, balancing the virulence and immunogenic properties of these proteins, which is essential for successful maintenance of long-term *M. tuberculosis* infection.

In conclusion, ESX-1 is a fascinating secretion system with many unique regulatory and functional characteristics. It is also one of the best-understood examples of virulence factors in *M. tuberculosis* complex. Upon deletion of RD1 or *esxAB* *M. tuberculosis* almost completely lost its virulence in animal models.

However, it fails to fulfill all the requirements for a molecular virulence factor as proposed by Falkow,¹⁹² as orthologous genes are present in some non-pathogenic mycobacteria (such as *M. smegmatis*).¹⁹³ This fact suggests that ESX-1 has evolved to be adapted to life inside eukaryotic cells. Recent results indicate that ESX-1 allows *M. tuberculosis* to escape from the phagosome. Importantly, there are still many proteins essential for ESX-1 function whose precise functional role or topological location still needs to be determined. In addition, ESX-5 allows the secretion of many PE and PPE proteins, in example PE_PGRS33. Disruption of the PE_PGRS33 encoding gene causes attenuation of *M. tuberculosis*.¹⁹⁴ The exact role of PE/PPE proteins on virulence, however, remains largely unknown (see section on “Proteins of Unknown Function”).

The operon espACD (Rv3616c-Rv3614c). EspA is a protein secreted by ESX-1 whose gene is outside of the RD1 region and, therefore, is present in BCG. EspC and EspD show significant homology to Rv3865 and Rv3867, respectively.¹⁸¹ Although its small size, EspC is secreted by ESX-1; the secretion of EspD, unlike EspA and ESXA, does not exclusively require the ESX-1 system. Evidence for stabilization of cellular levels of EspA and EspC by EspD has been presented, and depletion of EspD results in loss of ESXA secretion. Site-directed mutagenesis of *espD* reveals that its role in the maintenance of cellular levels of EspA in *M. tuberculosis* is distinct from its facilitation of ESXA secretion. The polycistronic nature of *espA*, *C* and *D* was evident when the virulence and ESX-1 function of an *M. tuberculosis* mutant in *espC* was only complemented with the entire *espA-espC-espD* gene cluster.¹⁸² In spite of this genetic relatedness, there are strong functional dissimilarities among proteins encoded in the *espACD* operon. EspA and EspC secretion require EspD, but EspD secretion does not require EspA, EspC or ESX-1.¹⁹⁵ EspC is a potent antigen in both active and latent TB infection. T-cell responses to EspC were highly specific (93%) for *M. tuberculosis* infection. The immunodominance of EspC, equivalent to that of ESAT6 and CFP10, and its high antigenic specificity make this protein a promissory TB vaccine candidate and a potential T-cell-antigen.¹⁹⁶

M. tuberculosis EspC and EspA mutants have shown a highly reduced capability to grow in BMDM.¹⁸² In addition, an *M. tuberculosis* H37Rv mutant in *espA* is also highly attenuated in lungs of C57BL/6 infected mice in terms of both bacterial load and pulmonary damage.¹⁸¹ The deletion of the entire *espACD* operon leads to a large reduction of *M. tuberculosis* H37Rv load in SCID mice.¹⁹⁷ It is still not clear if the impact of EspA, EspC and EspD on the virulence of *M. tuberculosis* is only due to their role in ESXA and ESXB secretion or if these proteins exert a direct interaction with the host. Regarding the versatility of this pathogen, this cannot be discarded and should be addressed.

The ESX-5 secretion system. MTBC species contain a large number of genes that encode for an *M. tuberculosis* unique family of proteins whose N-termini contain the characteristic motifs Pro-Glu (PE) or Pro-Pro-Glu (PPE). A subgroup of the PE proteins contains polymorphic GC-rich sequences (PGRS). The function of most of these proteins remains unknown and speculative. Abdallah et al. have shown that PE_PGRS proteins from *M.*

marinum are secreted by components of the ESX-5 (Rv1782-Rv1798) system, which is different from ESX-1, but also belongs to T7SS.¹⁹⁸ These observations, which now need to be addressed and confirmed in *M. tuberculosis*, have opened new perspectives on the function of these highly abundant proteins.¹⁹⁹ In contrast to ESX-1, the effector proteins secreted by ESX-5 are not required for the translocation of *M. tuberculosis* or *M. marinum* to the cytosol of host cells. These results reveal distinct roles for two different type VII secretion systems during infection and shed light on how virulent mycobacteria manipulate the host cell in various ways to replicate and spread.²⁰⁰ It has been reported that ESX-5 is essential for PE_PGRS33 export.²⁰¹ The PE domain of PE_PGRS33 (Rv1818c) is crucial for its surface localization, and it has been demonstrated that a PE domain lacking its first 30 amino acids loses its function. However, single amino acid substitutions in two extremely well-conserved regions within the N-terminal domain in all PE proteins had some effect on the stability of PE_PGRS33, but not on its localization.

Recently, Bottai et al.²⁰² have knocked out the *ESX-5* locus in *M. tuberculosis* H37Rv. Whereas the *M. tuberculosis* mutant in *Rv1794* displayed no obvious phenotype, the other four mutants (*eccA5*, *eccD5*, *ESXM* genes and the *ppe25-pe19* region) showed defects in secretion of the ESX-5-encoded ESXN and PPE41; the latter is a representative member of the large PPE protein family. Mutants in *eccD5* and *ppe25-pe19* are attenuated both in BMDM and in the SCID mouse infection model. These findings indicate an essential role of ESX-5 for transport of PPE proteins, cell wall integrity and full virulence of tuberculosis.

Type II secretion system. In bacteria, lipoproteins are exported via type II secretion systems or general Sec secretory pathway; however, a signal peptidase (the lipoprotein signal peptidase LspA) different from the canonical signal peptidase of SecII pathway cleaves the signal peptide of lipoproteins exported by this secretory pathway. The genome of *M. tuberculosis* potentially encodes for more than 90 lipoproteins (see above). When the *M. tuberculosis* *lspA* gene was inactivated by homologous recombination, lipoproteins were synthesized but not processed; although this gene is dispensable for in vitro growth, its deletion markedly affects the growth of mycobacteria in murine J774 macrophages and severely attenuates their virulence in BALB/c mice.¹²³ Interestingly, Rampini et al. have observed that the loss of LspA results in attenuation without affecting phagosome maturation arrest (a central point for *M. tuberculosis* survival in phagocytes) showing a low degree of co-localization with lysosomal-associated membrane protein 1 (LAMP1) and with acidic compartments.²⁰³

In conclusion, the genome of *M. tuberculosis* encodes a very large number of potential lipoproteins, largely contributing to cell envelope structure. It is surprising that *M. tuberculosis* is viable in vitro without properly modified lipoproteins while mutant lacking the signal peptidase LspA cannot survive in vivo. This property, together with the fact that lipoproteins are well-known TLR ligands that may influence the intracellular fate of the bacteria, highlights the role of lipoproteins in vivo. It has been observed that the genes for lipoprotein synthesis and modification are dispensable for in vitro growth of Gram-positive bacteria but are essential for Gram-negative bacteria. However, the essentiality of

LspA seems to depend on the mycobacterial specie since the gene encoding this protein could not be mutated in BCG (Sander P, personal communication). Also, the first enzyme in lipoprotein synthesis, prolipoprotein diacylglycerol transferase, is also essential in *M. tuberculosis* but not in *M. smegmatis*.²⁰⁴

Accessory SecA2 export system. Mycobacteria have two SecA proteins, SecA1 and accessory SecA2.²⁰⁵ SecA2 is a nonessential preprotein translocase ATPase. Contrasting with the canonical preprotein translocase ATPase SecA1, SecA2 exports a limited number of proteins. SecA2 is essential for the full virulence of *M. tuberculosis*, suggesting that one or more of the proteins it exports are important in pathogenesis.²⁰⁶

SecA1 and SecA2 have independent functions, since SecA2 cannot compensate for the lethal SecA1 deletion. Similarly, overexpression of SecA1 fails to rescue the phenotypes of a *secA2* deletion mutant. The analysis of proteins exported into the culture media (culture filtrates) by an *M. tuberculosis secA2* mutant has revealed that among the very few proteins absent exclusively in the mutant strain was the superoxide dismutase (SodA), which is an oxygen radical detoxifying enzyme. Therefore, this finding indicates that SodA depends on SecA2 for export despite the absence of a signal peptide.²⁰⁷ Another antioxidant enzyme, KatG, also lacks a signal peptide and is dependent on SecA2 for export. A more detailed description of the role of both SecA2 and SodA in the virulence of MTBC species is given below in this review (see section on “Inhibition of apoptosis”).

Proteins Inhibiting Antimicrobial Responses of the Macrophage

During infection, macrophages ingest and destroy pathogens, recruit other cells of the immune system, and present antigens from the microbe to cells of the adaptive immune system. However, pathogenic mycobacteria have evolved mechanisms to counteract the macrophage microbicidal ability, some of those mechanisms are: (1) increasing in the resistance to host toxic compounds, (2) arrest of the normal progression of the phagosome and (3) avoidance of the induction of apoptosis. In this section, we discuss the advance in knowledge of these survival mycobacterial strategies and described those virulence proteins involved in these process and the genes encoded by them.

Oxidative and nitrosative stresses. Resistance to macrophage-mediated killing is critical to the virulence of *M. tuberculosis*. Upon phagocytosis of bacteria, host cells produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) with potential bactericidal activity. These reactive oxygen/nitrogen intermediates (ROI/RNI) react with a wide variety of molecules, including nucleic acids, proteins, lipids and carbohydrates. Most of intracellular pathogens, including mycobacterial species, have developed a range of defense strategies to protect themselves against the damaging effects of these agents. However, for pathogenic mycobacterial species these strategies seem to be different to those of other intracellular bacteria. For instance, the classic defense mechanism OxyR is dysfunctional in all members of the MTBC.²⁰⁸ Yet, other mechanisms or processes compensate for the lack of a functional OxyR. In fact, several proteins directly

involved in these resistance responses have been identified and characterized in mycobacteria. In addition, the finding that several genes likely involved in cell envelope processes are also important in *M. tuberculosis* resistance to oxidative and nitrosative stresses¹¹⁴ suggests that the highly impermeable mycobacterial cell wall acts as a barrier for ROI and RNI. The present section focuses on proteins that, either directly or indirectly, are necessary for the MTBC species to counteract oxidative and nitrosative stresses, and that have also been shown to be relevant for the virulence of pathogenic mycobacterial species. These proteins and their mechanisms are schematized in **Figure 4**.

Acr Family. There are two members of the α -crystallin (Acr) family of molecular chaperones in *M. tuberculosis*:

Acr1, also called 16-kDa α -crystallin or HspX, is part of the DosR regulon, a genetic program of *M. tuberculosis* induced by conditions that inhibit aerobic respiration and prevent bacillus replication that regulates the expression of a large number of dormancy-associated proteins.²⁰⁹ HspX is a dominant protein present in old, stationary phase cultures, but undetectable during logarithmic growth of *M. tuberculosis*. *acr1* transcription is induced by exposure to hypoxia or nitric oxide in the context of the dormancy program of *M. tuberculosis*²¹⁰ and during the course of in vitro infection of macrophages.²¹¹ The role of HspX in *M. tuberculosis* virulence has been tested in vitro and in vivo, but the results are not conclusive. While Yuan et al. have reported that a Δ *acr* mutant in *M. tuberculosis* is significantly impaired for growth in both murine BMDM and THP-1 cells,²¹¹ Hu et al. have shown just the opposite: another Δ *acr* mutant in *M. tuberculosis* exhibits increased growth following infection of BALB/c mice and in both resting and activated BMDM.²¹² Consistent with the latter, overexpression of HspX results in reduced growth of BCG within mouse lungs and liver.²¹³ Based on these findings, it has been proposed that HspX plays an active role in slowing the growth of *M. tuberculosis*.²¹²

Acr2 is the other Acr family member. Its expression is induced by heat shock,²¹⁴ oxidative stress²¹⁵ and uptake by both human monocytes and monocyte-derived macrophages.²¹⁶ In addition, Acr2 is highly expressed during both acute and chronic infection in a mouse model. The deletion of the *acr2* gene fails to impair the growth of *M. tuberculosis* either in murine BMDM or in mouse organs.²¹⁷ However, a delay in the disease progression has been observed over a prolonged mice infection with an *M. tuberculosis* mutant in *acr2*, evidenced by a reduced weight loss and diminished size of lung lesions in animals infected with the mutant strain as compared with those infected with the parental strain.²¹⁷

Altogether, these findings indicate that both α -crystallins, HspX and Acr2, contribute somehow to the infection with *M. tuberculosis*. Infections of macrophages that produce limited oxidative and nitrosative bursts with *acr1* and *acr2* mutants are necessary to determine whether there is a connexion between the role of HspX and Acr2 as stress responders and their virulence properties.

Rv2136c, Rv2224c and PonA2. *M. tuberculosis* mutants with transposon insertions in *Rv2136c*, *Rv2224c* and *ponA2* displayed a hypersensitive phenotype to low pH, antibiotics, sodium dodecyl

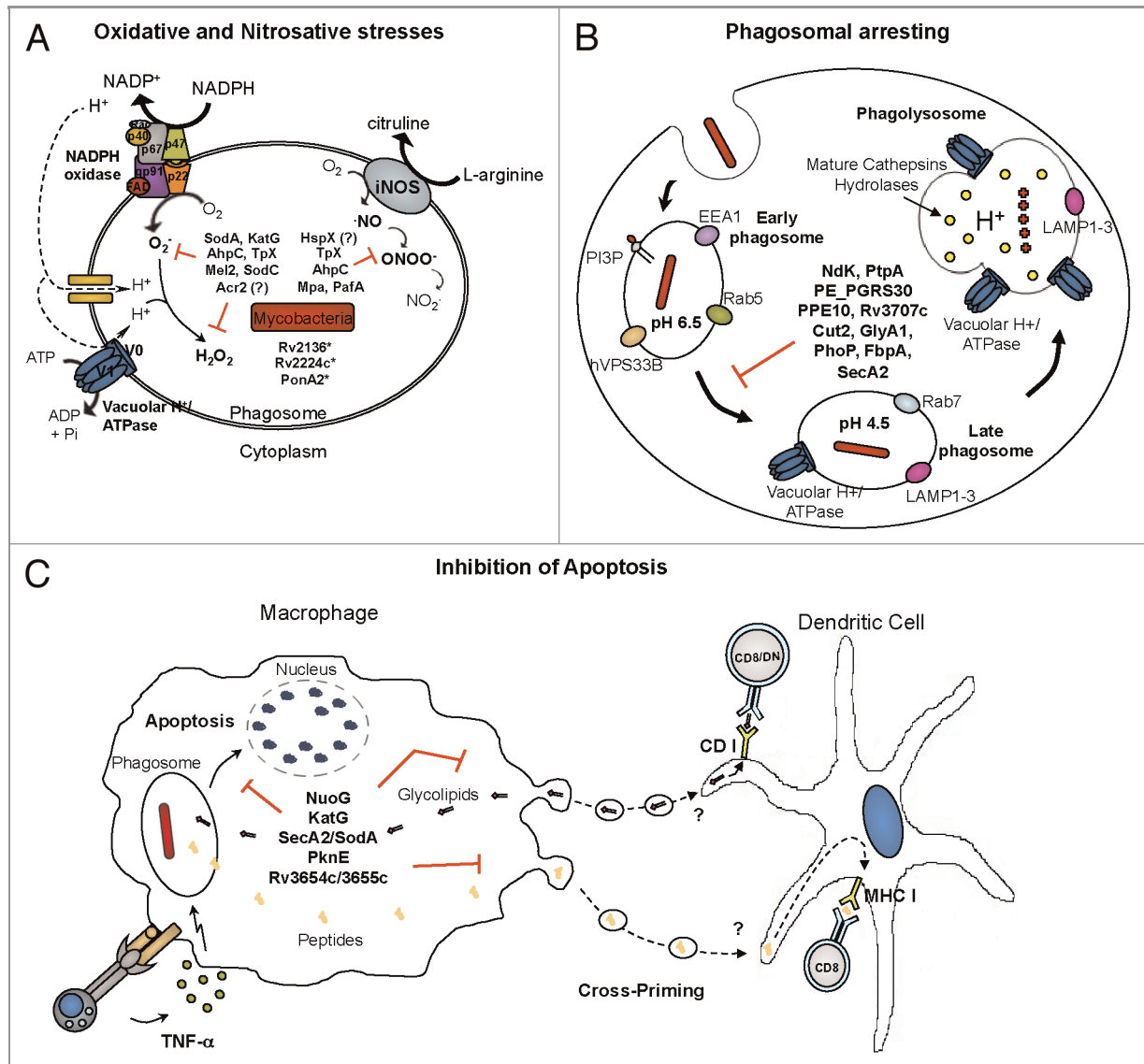


Figure 4. Antimicrobial properties of the macrophage. (A) Oxidative and nitrosative stresses, (B) phagosome arresting, (C) apoptosis and cross presentation. Proteins involved in the inhibition of antimicrobial mechanisms are indicated. *Proteins likely involved in cell wall associated resistance to oxidative burst.

sulfate, heat shock and reactive oxygen and nitrogen intermediates. These transposon mutant strains have also shown a variable level of attenuation when assayed C57BL/6 mice. While the *M. tuberculosis* H37Rv mutant in *Rv2136c* gene shows a remarkable impairment in its persistence in mouse lungs and spleen and markedly reduced gross pathology in lungs,¹¹⁴ the mutant in *Rv2224c* and *ponA2* shows attenuated phenotypes but not as strong as that detected in mutant $\Delta Rv2136c$. Although the function of *Rv2136c* gene is at present unknown, the close homology to an *E. coli* enzyme involved in the synthesis of peptidoglycan suggests an equivalent function in *M. tuberculosis*. PonA2 is predicted to have transglycosylase and transpeptidase activities, and thus likely to be also involved in peptidoglycan biosynthesis. Finally, *Rv2224c* encodes a putative carboxylesterase with lipase and proteinase activities (see sections on “Cell wall

proteins” and “ATP-dependent proteases”). These predicted functions for *Rv2136c*, *Rv2224c* and *PonA2* suggest that these proteins participate in cell wall processes, highlighting the importance of *M. tuberculosis* cell wall in protection against diverse stresses.

AhpC. AhpC is an alkyl hydroperoxide reductase C, an enzyme that reduces organic peroxides, similar to a family of bacterial and eukaryotic antioxidant proteins that directly reduces peroxides and peroxynitrites. Many lines of evidence have shown that AhpC is involved in the response to oxidative stress in mycobacterial species: in *M. tuberculosis*, as in most mycobacterial species, *ahpC* is located adjacent to *oxyR*, a central regulator of the peroxydative and nitrosative stress response that is dysfunctional in the MTBC species. The AhpC contribution to the peroxydative homeostasis of *M. tuberculosis* has been demonstrated in isoniazid resistance of

M. tuberculosis isolates that overexpress AhpC in the absence of catalase-peroxidase KatG (see below).²¹⁸ Therefore, *M. tuberculosis* compensates the lack of KatG catalase-peroxidase activity overexpressing AhpC.²¹⁹ Although AhpC is unlikely to confer resistance to rifampicin, its expression is upregulated by this antibiotic.²²⁰ Overexpression of AhpC in an *M. bovis katG* mutant strain has conferred resistance to both hydrogen peroxide and the organic peroxide cumene hydroperoxide.²²¹ AhpC crystal has been resolved²²² and its peroxynitrite reductase activity has been demonstrated in vitro.^{223,224} In addition, an *M. tuberculosis* mutant in *ahpC* showed higher susceptibility to peroxynitrite, but not to nitric oxide, than the wild-type strain.²²⁵ Importantly, this mutant strain also showed decreased survival in unstimulated macrophages, but the effect was no longer detectable upon IFN γ activation,²²⁵ suggesting that AhpC has an essential role in the resistance mechanisms to host oxidative agents only very early during the infection. Consistent with this latter finding, the knockdown of *ahpC* in *M. bovis* attenuates the virulence of the bacteria in a guinea pig model.²²¹ In conclusion, these findings clearly demonstrate that AhpC plays a role in the resistance of *M. tuberculosis* to oxidative stress; however, the contribution of this protein to bacterial virulence needs further investigation.

SodC. *M. tuberculosis* has two genes encoding superoxide dismutase (SOD) proteins, *sodA* and *sodC*. The function of Sod enzymes is the detoxification of ROS by conversion of O₂⁻ into molecular oxygen and hydrogen peroxide.²²⁶ SodC is a copper-containing SOD outer-membrane lipoprotein^{227,228} involved in the protection of *M. tuberculosis* against the extracellular superoxide generated by host cells. Consistently, *sodC* transcription is upregulated upon macrophage infection.²²⁹ Moreover, an *M. tuberculosis* mutant in *sodC* has shown very high susceptibility to superoxide generated externally, as compared with the wild-type strain.²³⁰ This mutant strain also showed decreased survival in IFN- γ -activated murine peritoneal macrophages and, importantly, the effect was no longer detectable in unstimulated macrophages or in activated macrophages from respiratory burst-deficient mice.²³⁰ Nonetheless, a mutant of *M. bovis* BCG in *sodC* did not show impaired growth in both activated and resting mouse BMDM.²³¹ One possible explanation for these apparently contradictory results could be the type of macrophages used in both studies. It has been proposed that murine-activated peritoneal macrophages produce higher amounts of ROS in response to infection than BMDM.²³⁰ Therefore, the lack of attenuation of the mutant strain in BMDM could be due to the absence of an effective oxidative burst. However, the fact that different genetic backgrounds were used to generate both *sodC* mutant strains cannot be overlooked. Paradoxically, an *M. tuberculosis* H37Rv mutant in *sodC* was virulent in guinea pig.²³¹ These controversial findings highlight the need for further research in different in vivo models to clearly establish SodC role in *M. tuberculosis* virulence.

Mel2. Mel2 is encoded by *mel2*, a member of a new class of bioluminescence-related genes present in numerous non-bioluminescent pathogens and symbionts. In a recent study by Cirillo et al. it has been shown that in *M. tuberculosis* Mel2 confers protection against ROI in vitro.²³² In this study, an *M. tuberculosis*

strain deleted in *mel2* gene was less persistent in both the lungs and spleen of C57BL/6 mice, with reduced pathology in these organs, as compared with its parental strain. The replication of the mutant strain was also significantly impaired in a murine macrophage cell line, in human peripheral blood monocyte-derived macrophages and in activated BMDM obtained from the wild-type C57BL/6 and oxidative burst defective (*phox*^{-/-} and *iNOS*^{-/-}) mouse strains. These attenuated phenotypes for the mutant strain in macrophages defective in the generation of either ROS or RNS provided evidence that *mel2* plays a role in resistance to both ROS and RNS in activated macrophages. However, when evaluated *iNOS*^{-/-} mice, the *mel2* mutant maintained an attenuated phenotype as compared with the wild type, while in *phox*^{-/-} mice the replication of the mutant in organs was similar to the wild type. These observations suggest that the primary mechanism by which Mel2 affects pathogenesis is through its ability to confer resistance to ROS.

KatG. KatG is the only enzyme with catalase-peroxidase activity that degrades H₂O₂ and organic peroxides in *M. tuberculosis*. KatG activates the anti-tuberculous drug isoniazid (INH), converting it to several reactive species that inhibits a mycolic acid biosynthetic enzyme,²³³ thus, almost of all the described INH resistant mutants bear mutations in this gene. In two independent studies, INH-resistant *M. tuberculosis* H37Rv strains that showed no KatG activity were attenuated in both mice (BALB/c and MHC class II-knockout mice) and guinea pigs, as determined by bacterial counts in organs or animal survival.^{234,235} In one of these reports, both the catalase activity and the virulence were restored by re-introduction of a wild-type copy of *katG*.²³⁵ Similarly, an INH-resistant and catalase negative *M. bovis* strain was attenuated in guinea pigs as compared with the same strain complemented with a functional copy of *katG*.²³⁶ Finally, an *M. tuberculosis* Erdman strain with a *katG* deletion was attenuated in wild-type C57BL/6 mice and *iNOS*^{-/-} mice, but not in gp91*phox*^{-/-} mice lacking the gp91 subunit of NADPH oxidase.²³⁷ Similar results have been reported in resting and activated BMDM obtained from the same mouse strains and in activated *iNOS*^{-/-} macrophages, therefore, suggesting that the main role of KatG in the virulence of *M. tuberculosis* is to catabolise the peroxydes generated by the phagocyte NADPH oxidase.²³⁷ Altogether, these results indicate that the catalase-peroxidase activity exerted by KatG is highly important for the virulence of *M. tuberculosis*.

TpX. TpX is a thiol peroxidase enzyme that catalyzes the reduction of hydroperoxides and peroxynitrite in *M. tuberculosis*. These enzymatic activities have been demonstrated in mycobacteria both in vitro using purified recombinant protein^{238,239} and also in vivo by comparative analysis of an *M. tuberculosis* mutant in *tpx* gene and its parental strain.²⁴⁰ In this latter study, Hu et al. have reported that the *M. tuberculosis tpx* mutant is more susceptible to oxidative and nitrosative stress, showing reduced peroxidase activity.²⁴⁰ Importantly, when tested in mice, the *tpx* mutant strain was less lethal and persistent than the parental strain. In macrophages, the *tpx* mutant strain showed impaired replication in both activating and resting macrophages, as compared with the wild-type strain. In contrast, the attenuated phenotype was not observed in the macrophages *iNOS*^{-/-}, which

produce a limited oxidative burst.²⁴⁰ Undoubtedly, the study mentioned above indicates that TpX is essential for protecting *M. tuberculosis* against the RNS and ROS produced by the host cells during tuberculosis infection.

Enzymes that directly participate in the RNI and ROI detoxification mechanisms such as AhpC, SodC, KatG and TpX are essential for the full virulence of the MTBC species. However, virulence proteins with non-conserved detoxification activities have also shown to confer to the bacteria protection against oxidative and nitrosative stresses, indicating that MTBC contain additional antioxidant mechanisms to respond to the hostile environment in the phagosome and phagolysosome.

Some of these non-classical oxidative and nitrosative stress response virulence proteins, such as Rv2136c, Rv224c and PonA2, are likely implicated in cell wall integrity, suggesting that the mycobacterial lipid-rich cell envelope could act as an effective barrier against the entry of RNI and ROI. Other proteins of this class are the two members of α -crystallin family, HspX and Acr2, molecular chaperones primarily described as proteins expressed in multiple stress conditions. Although the role of these mycobacterial chaperones in the in vivo essential antioxidant mechanism is still unclear, the current knowledge of their eukaryotic counterpart may allow to make some conjectures about their function: it has been described that the eukaryotic heat shock protein 90 (Hsp 90) and α -crystallin protect some peptidase activities of the active form 20 S proteasome against oxidative inactivation.²⁴¹ In turn, authors of several studies have reported that the proteasome plays a crucial role in the selective recognition and degradation of oxidized proteins.²⁴² Therefore, a possible role of HspX and Acr2 in nitrosative and oxidative stresses may be through their interaction with the mycobacterial proteasome. In connection with the latter, the transcriptional regulator ClgR positively regulates the expression of Acr2 together with a number of proteases, thus, supporting a role of Acrs in protein homeostasis (see section on "Proteases").

Phagosome arresting. A phagosome is a cellular compartment in which pathogenic microorganisms can be killed and digested. Phagosomes fuse with lysosomes in their maturation process, forming phagolysosomes. Some intracellular bacterial pathogens reside inside phagosomes and either divide or grow inside of the formed phagolysosome or escape into the cytoplasm before the phagosome fuses with the lysosome. Many mycobacteria manipulate the host macrophage to prevent nitrous acid-containing lysosomes from fusing with phagosomes and creating mature phagolysosomes. Therefore, one of the main mechanisms elicited by intracellular mycobacteria to survive and replicate inside the host cells is to arrest the normal process of phagosome maturation, which enables bacterial survival in a non-acidified intracellular compartment. The identification of mycobacterial components that interfere with maturation of the phagosomal compartment after ingestion has become a central issue of investigation in tuberculosis.

Based on microarray-based screening of a transposon library, Stewart et al. have found that *ppe10*, *Rv3707c*, *cut2* and *glyA1*, among other genes, were relevant for *M. bovis* BCG to arrest phagosome acidification following uptake by macrophages. *cut2*

encodes a protein member of a family of serine esterases and *glyA1* encodes a protein member of a family of serine hydroxymethyltransferases. *ppe10* encodes a member of PPE family and *Rv3707c* encodes a conserved hypothetical protein (CHP). Transposon mutants in these genes have shown reduced replication inside macrophages as compared with the parental BCG strain, being these attenuations most pronounced at later point times.¹⁰¹ These findings highlight the importance of avoiding phagosomal acidification for the intracellular survival and persistence of pathogenic mycobacteria.

Other mycobacterial proteins involved in arresting macrophage phagosomal maturation with a clear role in the virulence of pathogenic *Mycobacterium* species are Ndk, PtpA and PE_PGRS30.

These proteins and their mechanisms are schematized in **Figure 4**.

Ndk. *ndk* encodes a nucleoside diphosphate kinase with ATP and GTP binding activity as well as hydrolysis activity, demonstrated in vitro.²⁴³ It has also been shown that this protein is autophosphorylated and secreted into the culture medium by *M. tuberculosis*, and that purified Ndk in combination with ATP produces cytotoxicity in macrophages.²⁴³ Recently, Sun et al. have shown that recombinant Ndk dephosphorylates the cellular Rab7-GTP and Rab5-GTP in a cell-free biochemical assay, resulting in the deactivation of these enzymes. Moreover, the authors have found evidence suggesting that Ndk inhibits the recruitment of Rab5 and Rab7 effectors to phagosomes (early endosome antigen 1-EEA1 and Rab7-interacting lysosomal protein, respectively). Based on those results, it has been proposed that, within the phagosome, Ndk released from pathogenic mycobacteria might have access to the cytosolic face of the phagosomal membrane so as to interact with and inhibit effectors of phagosome maturation. Consistently, infection of macrophages with a *ndk* knock-down *M. bovis* BCG (Pasteur 1173P2) strain resulted in increased fusion of its phagosome with lysosomes along with significantly decreased survival of the mutant.²⁴⁴

PtpA. PtpA is a low-molecular weight tyrosine phosphatase²⁴⁵ reported to dephosphorylate VPS33B, a host protein involved in the regulation of membrane fusion within the endocytic pathway. This phosphatase activity resulted in arrested phagosome maturation by *M. tuberculosis*. Later on, it has been found that PtpA binds to subunit H of the macrophage vacuolar-H⁺-ATPase machinery, a multisubunit protein complex in the phagosome membrane that drives luminal acidification. This interaction and dephosphorylation of VPS33B are both required for PtpA inhibition of macrophage phagosome-lysosome fusion and phagosome acidification.²⁴⁶ Consistent with this proposed role for PtpA in arresting phagosomal maturation, an *M. tuberculosis* H37Rv mutant in *ptpA* has shown impaired replication in the human THP-1 macrophage cell line.²⁴⁷ However, the knockout of *ptpA* in *M. tuberculosis* Erman strain showed no apparent variation in the bacterial replication in mouse organs compared with the wild type.²⁴⁸ The two different infection models used in these studies could explain these opposite findings.

PE_PGRS30. PE_PGRS30 is a member of the PE_PGRS family. In a very recent study by Iantomasi et al. it was shown that

PE_PGSR30 is essential for the full virulence of *M. tuberculosis* in both mice and macrophage models. These authors found that the elimination of PE_PGSR30 from *M. tuberculosis* H37Rv results in an attenuated strain in terms of replication in mouse organs and lung pathology. The mutant strain showed impaired replication in mouse lungs during the chronic steps of infection and this phenotype was correlated with reduced tissue damage in lungs of infected BALB/c mice. When assessed in human THP-1 and murine J774 macrophages, the mutant also showed reduced replication in both cell lines as compared with that of the wild-type strain. Most importantly, this protein seems to have a role in the phagosomal arresting strategies developed by *M. tuberculosis* after macrophage uptake, since the mutant strain showed higher colocalization with a lysosomal marker. This finding strongly suggests that the inability of the mutant strain to inhibit phagosome-lysosome fusion is the reason of the attenuated phenotype observed in the in vivo and ex vivo models. In conclusion, the study of Iantomasi et al. has characterized one of the first PE_PGSR proteins with a certain role in the virulence of *M. tuberculosis*.²⁴⁹

Likely, the most efficient strategy used by MTBC to counteract the macrophage antimicrobial actions is to subvert the normal progression of the phagosomal compartment and prevent it from maturing into an active phagolysosome. This modulation of the intracellular endosomal trafficking allows the bacteria to stay in a non-acidic hospitable niche suitable for replication and helps to avoid its immunological detection. Although the process of phagosomal arresting induced by *M. tuberculosis* is not fully understood, some aspects of this process are coming into sight. The endosomal tethering molecule EEA1 is a specific Rab5 effector that plays an essential role in phagosomal maturation.²⁵⁰ EEA1 associates with phosphatidylinositol 3-phosphate (PI3P) in endosomal membranes via its PI3P binding FYVE domain, and it has been demonstrated that this association is essential for phagosome maturation since inhibition of PI3P production arrests the process. *M. tuberculosis* prevents EEA1 recruitment to phagosomes in infected macrophages, thus precluding phagolysosome formation.²⁵¹ Besides, *M. tuberculosis* inhibits Ca²⁺ signaling, which together with calmodulin are necessary for recruitment of PI3PK responsible for the production of PIP3 in the endosomal membranes. Therefore, one possible mechanism by which *M. tuberculosis* prevents EEA1 recruitment to phagosomes is through inhibition of Ca²⁺ efflux.²⁵² However, a reduced level of Ca²⁺ in macrophages infected with *M. tuberculosis* is not sufficient to completely explain the phagosome maturation blocking in these infected cells, suggesting that other steps of this phagosomal maturation pathway are targeted by this pathogen. Vergne et al. found that *M. tuberculosis* culture supernatant proteins dephosphorylate PIP3P in vitro and that this PIP3P phosphatase activity prevented the in vitro phagosome-lysosome fusion. The authors identified the secreted acid phosphatase M (SapM) as one of the culture supernatant proteins responsible of this PIP3P dephosphorylation.²⁵² However, further studies have shown that a mutant in *sapM* gene of *M. bovis* BCG fails to prevent phagosome arresting, therefore, suggesting that other phosphatases could compensate the lack of SapM from BCG.²⁵³ In addition to PIP3P,

other proteins (Rab7, Rab5 and the vacuolar protein VPS33B) have been also proposed as potential target of phosphorylation/desphosphorylation mediated by *M. tuberculosis*. In this regards, the mycobacterial kinase Ndk has been demonstrated to phosphorylate Rab7 and Rab5 in vitro.²⁴⁴ Besides, PtpA contributes to the inhibition of phagosome acidification induced by *Mycobacterium* spp and this PtpA action is dependent on its binding to the H subunit of the vacuolar ATPase and its phosphatase activity on VPS33B.²⁴⁶ It has been proposed a model in which PtpA inhibits the vacuolar ATPase trafficking to the mycobacterial phagosome. In this model, PtpA is secreted by *Mycobacterium* into the host cytosol, binds to subunit H of the vacuolar ATPase complex, disrupting the interaction between the two protein complexes and localizing itself near to VPS33B. PtpA then dephosphorylates and inactivates VPS33B; consequently, the normal progression of membrane fusions is blocked.²⁴⁶

Proteins involved in the biosynthesis of cell wall lipids, such as PhoP²⁵⁴ and Ag85A,²⁵⁵ also have a role in the phagosome arresting exerted by MTBC. Most likely, these proteins are not direct effectors of phagosome trafficking, instead they participate in the synthesis of compounds that are actually implicated in this cellular process. For instance, the synthesis of cell wall TDM and the SL is regulated by the two-component system PhoP/PhoR and these lipids have been described as implicated in blocking phagosome/lysosome fusion.²⁵⁶

The *M. tuberculosis* SecA2 system, a specialized protein export system, is also required for phagosome maturation arrest,²⁵⁷ suggesting that there are effectors of phagosome maturation that are exported into the host environment by this system. However, one important question that remains unclear is how mycobacterial secreted proteins gain access to the host cytoplasm and thereby to their endosomal trafficking pathway targets. The bacterial escape from endosomal compartment to the cytoplasm has been demonstrated in the infection of macrophages with *Mycobacterium marinum*²⁵⁸; however, for MTBC this point is still matter of discussion.²⁵⁹

Inhibition of apoptosis. The programmed cell death or apoptosis is one of the major mechanisms of the innate immune response elicited by eukaryotic organisms against pathogens. In this process, the host controls the infection at the expense of killing infected cells, but favoring efficient cytotoxic T cell priming via the detour pathway of antigen cross-presentation.²⁶⁰ Accordingly, viral, protozoan and bacterial pathogens have developed anti-apoptotic capacities to counteract this host microcidal activity. It has been extensively reported in the literature that *M. tuberculosis* induces apoptosis upon infection of host cells.²⁶¹⁻²⁶³ However, the magnitude of the apoptotic response varies depending on the MTBC strain that infects the cell. In addition, a negative correlation between the bacterial virulence and its capacity to induce apoptosis has been found.^{261,264} Thus, *M. tuberculosis* infection mainly results in necrosis, whereas attenuated mutant infections primarily induce apoptosis.²⁶⁵⁻²⁶⁷ Recent studies have reported the identification and characterization of several anti-apoptotic *M. tuberculosis* genes, specifically *nuoG*, *katG*, *sodA/secA2*, *pknE* and *Rv3654c/Rv3655c*. It is not surprising that most of these genes play roles in

the bacterial redox homeostasis, since phagosomal ROS, which are generated after *M. tuberculosis* infection, trigger the induction of apoptosis.²⁶⁶

In this section, we describe those anti-apoptotic factors that in turn have been shown to be essential for the full virulence of *M. tuberculosis* species (Fig. 4).

NuoG. NuoG is one of the 14 subunits of the type I NADH dehydrogenase, NADH-1, and the gene that encodes it was first described by Velmurugan et al. as an anti-apoptotic gene in a gain-of-function screening for anti-apoptotic *M. tuberculosis* genes performed in *M. smegmatis*. Its function was then confirmed using an *M. tuberculosis* strain deleted in *nuoG*.²⁶⁸ The mutant induced apoptosis in human THP-1 cells and cultured primary mouse macrophages, while the wild-type strain did not. Importantly, both BALB/c and SCID mice inoculated with the mutant strain survived longer than those inoculated with the wild-type strain. Furthermore, in the lungs of BALB/c mice, the bacterial load of the mutant was smaller than that of the wild-type strain.²⁶⁸ Thus, these findings are in line with previous evidence indicating that there is a positive correlation between virulence and inhibition of apoptosis. Recently, the same research group demonstrated that NuoG is involved in inhibiting an extrinsic TNF- α -dependent apoptosis pathway.²⁶⁹ Furthermore, they have found that the pro-apoptotic phenotype of the *nuoG* mutant is ROS-dependent, since in murine BMDM derivative and primary human alveolar macrophages apoptosis was abolished in the presence of both ROS scavengers and in the absence of a functional cellular NADPH oxidase system (NOX2).²⁶⁹ In addition, it has been recently reported that NuoG also suppresses neutrophil apoptosis and that the lack of *nuoG* in *M. tuberculosis* accelerates CD4 T cell priming, suggesting that inhibiting neutrophil apoptosis delays adaptive immunity in tuberculosis.²⁷⁰

SecA2/SodA. *secA2* encodes a preprotein translocase ATPase. SecA2 is a putative new type of secretion pathway that translocates superoxide dismutase A (SodA) and some others proteins to the culture supernatant (see section 3, Inhibition of apoptosis).^{206,271} In two different studies, the *secA2* deletion in *M. tuberculosis* H37Rv displayed an attenuation of the bacterial replication in organs of immunocompetent C57BL/6 mice.^{206,272} One of these studies has also shown longer survival of both C57BL/6 and SCID mice infected with the mutant strain as compared with animals infected with the wild-type strain.²⁰⁶ In addition, the mutant was attenuated in resting BMDM from both wild-type and *phox^{-/-}* (deficient in oxidative burst) mice, but not in activated wild-type macrophages.²⁷² These results indicate that SecA2 contributes to intracellular growth even in the absence of an oxidative burst.

In another study, Hinchey et al. have demonstrated that SecA2 prevents apoptosis in BMDM since an *M. tuberculosis* mutant in *secA2* has induced higher macrophages apoptosis than its parental strain.²⁷³ Based on the activity of different caspases (2, 8 and 9) in the mutant and wild-type strains, these authors have suggested that the lack of SecA2 induces the initiation of apoptosis through both the extrinsic and intrinsic caspase-dependent pathways. Importantly, the pro-apoptotic phenotype of macrophages infected with the mutant in *secA2* was reverted with the

introduction of SecA2-dependently secreted *sodA* gene, indicating that secretion of SodA is likely to be the major SecA2-dependent process involved in the inhibition of host cell apoptosis.²⁷³ Consistently, it has been reported that the mutation of *sodA* in *M. tuberculosis* H37Rv conferred high susceptibility to hydrogen peroxide and attenuation in a mice model (see the section on "Oxidative and nitrosative stresses"). In addition, the mutant in *sodA* induced higher apoptosis in mouse lungs than the wild type.²⁷⁴

Finally, it has been recently reported that SecA2 is required for phagosome maturation arrest.²⁵⁷ In this study, it has been shown that shortly after infection, phagosomes containing an *M. tuberculosis* mutant in *secA2* are more acidified and show a higher association with markers of late endosomes than phagosomes containing the wild-type strain. These results suggest that the phagosome maturation arrest defect of the mutant is responsible for the intracellular growth defect. Altogether, these results indicate that SecA2 secretion system, most likely through SodA, inhibits extrinsic and intrinsic apoptosis pathways induced upon *M. tuberculosis* macrophages infection, in a mechanism probably independent of oxidative burst. The in vivo attenuation detected in *secA2* *M. tuberculosis* mutants are likely to be due to the capacity of SecA2 to avoid apoptosis and antigen-specific CD8⁺ cross-presentation²⁷³ as well as the capacity of altering the intracellular trafficking in favor of the bacteria.

PknE. *pknE* encodes the serine/threonine kinase E, PknE. Jayakumar et al.'s study has shown that the deletion of *pknE* from *M. tuberculosis* results in increase in nitric oxide-mediated apoptosis in human THP-1 macrophages and decrease in production of pro-inflammatory cytokines, TNF- α and IL-6.²⁷⁵ In vitro assays of the same research have also shown that the mutant is more resistant to nitric oxide donors than the wild-type strain. Altogether, PknE may inhibit apoptosis by eliminating reactive nitrogen species that would be indispensable for the TNF- α -mediated induction of apoptosis. The only assessment of the essentiality of *pknE* for *M. tuberculosis* virulence, done in human THP-1 cells, has shown that a mutant in *pknE* displays a moderate impairment in the intracellular replication at late points of infection.²⁷⁵ In this regard, further in vivo studies are needed to establish the role of oxide-mediated apoptosis induced by PknE in the virulence of *M. tuberculosis*.

Rv3654c and Rv3655c. The role of *Rv3660c-Rv3654c* operon in apoptosis inhibition was initially demonstrated after screening for the lack of ability to inhibit macrophage apoptosis of an *M. tuberculosis* H37Rv transposon library.²⁷⁶ This study has also revealed that the proteins encoded in this operon affect mainly the extrinsic apoptosis pathway, since significant suppression of caspase-8 activation (part of extrinsic apoptosis cascade) was observed in macrophages infected with the wild-type strain, but not in those infected with the mutant strain. The virulence of this mutant strain has been evaluated in both apoptotic (detached macrophages) and non-apoptotic macrophages (attached macrophages), showing that the mutation impairs the replication of *M. tuberculosis* in apoptotic macrophages, but not in those non-apoptotic.²⁷⁶ These findings suggest that the products of *Rv3660c-*

Rv3654c operon are somehow important for the virulence of *M. tuberculosis*.

Further experiments using macrophages transfected with each gene of the *Rv3660c-Rv3654c* operon indicate that *Rv3654c* and *Rv3655c* are the proteins responsible for the extrinsic inhibition of apoptotic macrophage response.²⁷⁶ Despite these important advances, more research is necessary to confirm the role of *Rv3654c* and *Rv3655c* in the virulence of *M. tuberculosis* and to define the precise role of these proteins in the manipulation of cell apoptosis responses.

Apoptosis is an innate defense mechanism by which the host eliminates the niche for bacterial growth. In addition, increasing numbers of publications have shown that apoptosis of infected macrophages produces vesicles containing bacterial antigens that can be engulfed by dendritic cells to prime antigen-specific T cells.²⁷⁷ Therefore, apoptosis would promote the induction of the adaptive immune response apart from its role in innate immunity.

There are alternative pathways by which the apoptosis is triggered, including the apoptosis induced by granzyme B, the lysosomal pathway,²⁷⁸ and the extrinsic and intrinsic apoptotic pathways. In the extrinsic pathway, the binding of TNF- α and FasL ligands to their receptors triggers apoptosis.²⁶⁶ In the intrinsic apoptotic pathway the mitochondria releases cytochrome c and other factors from the mitochondrial intermembrane space that promote apoptosis.

The generation of ROS in phagosome containing bacteria by the action of NOX2, is one of the mechanisms by which infected macrophages induce TNF- α -mediated apoptosis.

The concept that virulent mycobacteria modulate the cell death program inhibiting apoptosis and favoring necrosis is gaining acceptance in the research community. It has been proposed that *M. tuberculosis* affects different cell signaling pathways to inhibit macrophage apoptosis. One of the anti-apoptotic strategies used by mycobacteria is to control the production of ROS.²⁶⁶ In this section we describe two mycobacterial proteins involved in this ROS dependant-anti-apoptotic mechanism: NuoG subunit of the type I NADH and SecA2, a protein required for the secretion of the superoxide dismutase SodA, among other proteins. On the other hand, the mycobacterial PknE inhibits macrophages TNF- α -mediated apoptosis through elimination of RNS, indicating that RNS also participate in the cellular death signaling. Finally, the precise mechanism by which proteins encoded in *Rv3660-3654c* operon inhibit the TNF- α -mediated apoptosis remains to be deciphered.

Other models describing how *M. tuberculosis* inhibits apoptosis favoring cell necrosis have been proposed: Lee et al.²⁷⁹ have demonstrated that after reaching a high intracellular load, virulent bacilli trigger a necrotic mode of macrophage cell death, releasing them to infect new host cells. In addition, *M. tuberculosis* can modify the surface of infected macrophages preventing completion of the apoptotic envelope and favoring a necrotic cell death outcome.²⁸⁰ Moreover, it has been shown that *M. tuberculosis* infection prevent macrophage cell membrane repair, which is important for the induction of apoptosis, predisposing the induction of cellular necrosis.²⁸¹ The study of Chen et al. provides a model by which *M. tuberculosis* manipulates the

apoptosis/necrosis cellular balance. Their study showed that virulent *M. tuberculosis* negatively modulates the production of prostaglandin E2 (PGE2) in infected macrophages.²⁸² PGE2 induces plasma membrane repair and prevent mitochondrial damage, protecting infected macrophages against necrosis. They also reported that virulent mycobacteria induce the production of LXA4 lipoxin, which is generated by 5- and 15-lipoxygenases (5- and 15-LO)²⁸² and that necrosis is positively regulated by LXA4 through inhibition of PGE2 synthesis. Based on these findings, together with the fact that 5 LO^{-/-} mice are significantly more resistant to tuberculosis infections,²⁸² Chen et al. have proposed that lipoxin production is involved in macrophage necrosis and in inhibition of the apoptosis induced by virulent mycobacteria. However, to completely elucidate these cell death signaling in the context of tuberculosis infections, it is necessary to identify the bacterial anti-apoptotic factors involved in these pathways and to understand the mechanism by which these factors affect the apoptotic/necrotic pathways.

Protein Kinases

Reversible protein phosphorylation is one of the principal signal-transduction pathways by which both eukaryotic and prokaryotic cells regulate the metabolism in response to external stimuli. In bacteria, signal transduction events are performed by two-component regulatory systems and by specific protein kinases and protein phosphatases.

M. tuberculosis genome encodes 11 eukaryotic-like serine-threonine protein kinases (PknA to PknL, except for PknC). All of these *pkn* genes have been shown to encode functional serine-threonine kinases and some of them have assigned roles in the modulation of different cellular events such as environmental adaptation, differentiation and cell division. These kinase proteins are mainly localized in membrane cell and cell wall of *M. tuberculosis*, but PknG is predominantly found soluble in cytoplasm.²⁸³ Only four of the 11 *M. tuberculosis* kinases (PknA, PknB, PknG and PknL) are conserved in *M. leprae*,²⁸⁴ as well as in *Corynebacterium glutamicum*, a more distantly related actinomycete.²⁸⁵ PknA, PknB and PknG have been also predicted to be essential in *M. tuberculosis*.^{286,287} As the massive genome decay shown by *M. leprae* suggests that only essential genes (coding for functional proteins) have been left unmutated; therefore, the essentiality of *pknL* in *M. tuberculosis* should be analyzed.

PknA and PknB are encoded in the same operon as genes involved in cell wall synthesis. Consistently, increasing evidence indicates that PknA and PknB play important roles in determining cell shape, morphology, and cell division.²⁸⁷⁻²⁸⁹ In addition, enzymes involved in *M. tuberculosis* peptidoglycan and mycolic acid biosynthesis are substrates of PknA and PknB,²⁹⁰⁻²⁹³ supporting the idea that these protein kinases modulate cell morphology and division in response to environmental cues through regulation of specific pathways involved in cell wall skeleton synthesis. Similarly, PknF is directly or indirectly involved in the regulation of cell growth and septum formation in *M. tuberculosis* as well as in glucose transport²⁹⁴ and also in the

regulation of the mycolic acid biosynthesis through phosphorylation of the β -ketoacyl-acyl carrier protein synthase III (FabH).²⁹³ It has been reported that PknF phosphorylates Rv1747, an ABC transporter, and this protein modification seems to be relevant for the replication of *M. tuberculosis* inside macrophages. However, a mutant deleted in *pknF* replicates normally in macrophages, indicating that other kinases compensate the lack of PknF-mediated phosphorylation in Rv1747 protein.²⁹⁵ PknI and PknK also seem to have a potential role in growth regulation of *M. tuberculosis*: The lack of *pknI* results in an increased growth of this bacterium within macrophages and a hypervirulence phenotype in severe combined immunodeficiency mice.²⁹⁶ Similarly, the absence of *pknK* displays an increased replication rate both in vitro and in immunocompetent mice²⁹⁷ compared with its parental strain. It has been proposed that VirS, a regulator of mono-oxygenase (*mymA*) operon, and four other proteins encoded by the *mymA* operon are potential substrates for PknK.²⁹⁸ In addition, *pknK* deletion from *M. tuberculosis* resulted in altered colony morphology and in increased resistance to acidic pH, hypoxia, oxidative and stationary-phase stresses in vitro.²⁹⁷

In *M. tuberculosis*, PknH phosphorylates InhA, a key enzyme of the fatty-acid synthase II system involved in mycolic acid biosynthesis,²⁹¹ and EmbR, a putative transcriptional regulator of the *embCAB* operon.²⁹⁹ The *embCAB* operon encodes arabinosyl-transferases involved in the biosynthesis of arabinogalactan and lipoarabinomannans.³⁰⁰⁻³⁰² Studying the putative substrate of PknH, Chao et al. have recently demonstrated a case of convergence of the two major signaling systems in *M. tuberculosis*: the two-component systems and serine-threonine protein kinases. These authors have found that PknH phosphorylates DosR, which in turns regulates the transcription of hypoxia and NO-inducible dormancy (DosR) regulon.³⁰³ Importantly, *pknH* deletion from *M. tuberculosis* induces hypervirulent phenotype in BALB/c mice in terms of bacterial load in mouse organs.³⁰⁴ Therefore, similarly to PknI and PknK, PknH seems to mediate the growth rate of *M. tuberculosis*, but unlike these Pkns, mutation in *pknH* has shown an unaltered in vitro and intracellular growth of *M. tuberculosis*. Evenmore, the *pknH* mutant replicated less in non-stimulated human macrophage cell line than the wild-type and complemented strains.³⁰⁴ This result may indicate that PknH controls the intracellular growth of *M. tuberculosis* through a signaling pathway that requires activation with external stimuli. In fact, it has been demonstrated that in the absence of PknH, *M. tuberculosis* is more resistant to nitric oxide in vitro; thus, PknH probably senses free radicals produced in response to activation of the host cells that contribute to its survival.³⁰⁴ Altogether, these results suggest that some protein kinases mediate the signaling that slow down the growth of intracellular *M. tuberculosis*. Uncontrolled replication inside the host cell may be disadvantageous if it contributes to rapid death of the host.

Regarding the function of PknJ and PknL, recent studies suggest that the glycolytic enzyme pyruvate kinase A (mtPykA)³⁰⁵ and Rv2175c, a DNA binding protein,³⁰⁶ are potential substrates of PknJ and PknL, respectively. Interestingly, *pknJ* is only conserved in MTBC genomes,³⁰⁷ suggesting a role of PknJ in the adaptation of MTBC species to the intracellular life.

The *M. tuberculosis* infection process involves cross-talk signals between the host and the bacterium; which results in the reprogramming of cell events in both organisms. Therefore, it is expected that protein kinases, as key components of the signal transduction pathways of *M. tuberculosis*, play key roles in the signaling network that allow *M. tuberculosis* to survive in the aggressive microenvironment of the host. Supporting this hypothesis, three members of the serine-threonine kinase family, PknD, PknE and PknG, have been shown to be required for the survival and persistence of *M. tuberculosis* inside hosts (see below). *PknD*. It has been proposed that PknD phosphorylates MmpL7,³⁰⁸ a transporter of the RND family essential for *M. tuberculosis* virulence⁴⁵ (see "Lipids and Fatty Acid Metabolism") as well as Rv0516c.³⁰⁹ Some evidence have suggested that Rv0516c is an anti-sigma factor antagonist (or anti-anti sigma factor) that regulates the expression of sigma factor SigF of *M. tuberculosis* in response to stress signals.³¹⁰ Greenstein et al. have found that Rv0516c is phosphorylated by PknD in a Thr residue.³⁰⁹ Based on these finding, together with the fact that SigF is essential for the full virulence of *M. tuberculosis*, it is plausible to speculate that PknD transduces environmental signals by controlling expression of specific groups of genes that are relevant for adaptation to the environment during infections. Consistently, it has been demonstrated that PknD is essential for invasion of mice brain endothelia by *M. tuberculosis*. An *M. tuberculosis* CDC1551transposon mutant in *pknD* has shown impaired invasion and survival in brain microvascular endothelial cells, but not in activated murine J774 macrophages, epithelial A549 cells, and umbilical vein endothelia. In addition, this mutant strain replicated less in brain than the wild type when inoculated in BALB/c mice,³¹¹ indicating that PknD may be a key factor required for central nervous system tuberculosis.

PknE. PknE seems to play a role in the virulence of *M. tuberculosis*, since it contributes to the persistence of *M. tuberculosis* in human macrophages cell line via an anti-apoptotic mechanism (see the section on "Inhibition of apoptosis").

PknG. The *pknG* gene is in a putative operon containing *glnH*, a gene encoding a protein potentially involved in glutamine uptake. Nevertheless, whether or not PknG is implicated in the regulation of glutamate metabolism of *M. tuberculosis* is controversial. While Cowley et al. have found that mutation of *pknG* alters the level of glutamate/glutamine in *M. tuberculosis*,³¹² in another study Nguyen et al. have reported that an *M. bovis* BCG mutant in *pknG* showed no differences either in the intracellular level of glutamine or in the uptake of glutamine.³¹³

It has been reported that PknG is unique in the fact that it undergoes autophosphorylation on Thr residues located at the N-terminus. Although this autophosphorylation seems unrelated to the regulation of its kinase activity, it is essential for the prevention of *M. bovis* BCG trafficking to lysosomes and for the bacterial survival in murine BMDM.³¹⁴

An *M. tuberculosis* H37Rv mutant strain in *pknG* gene was attenuated in lungs, spleen and liver of immunocompetent BALB/c mice, but not in those of CD-1 mice. This mutant also caused delayed mortality in SCID mice. Surprisingly, *pknG* mutation has been shown to produce some defects in the in vitro growth of *M.*

tuberculosis,³¹² but not in that of *M. bovis* BCG.³¹³ Therefore, further confirmative analysis should be performed to consider PknG as a virulence factor.

Pkns together with two-component systems and sigma factors are the constituents of the mycobacterial machine that regulates the adaptive gene expression in response to external stimuli. Increasing evidence suggest a crosstalk among all components of these regulatory network. For instance, it has been demonstrated that PknH phosphorylates DosR, a member of a two-component system, and that various sigma factor regulators are substrates of Pkns.³⁰⁹

Although the signaling pathways and endogenous substrates remain to be clearly established, the functional roles have been defined for most of the Pkns. PknA, PknB, PknF, PknI and PknK mediate the signaling that slows down the in vitro growth of *M. tuberculosis*. Moreover, PknI seems to play a role in the intracellular growth regulation of *M. tuberculosis*, suggesting that the uncontrolled replication inside the host cell may be disadvantageous if it contributes to a rapid death of the host. Similarly, the lack of PknH or K significantly increases the replication of *M. tuberculosis* in immune competent mice. However, whether these Pkns regulate the mycobacterial growth in animal models is still uncertain. This is mainly because of the difficulties to accurately determine the rate of bacterial replication in organs, as an increase in bacterial counts might be due to an increment of the growth rate but also to any adaptive advantage in the hostile intracellular environment. In addition, potential roles in central carbon metabolism and in cell wall biosynthesis have been assigned to PknG, F, J, L and PknA, B, H, D, F, respectively.

As mentioned above, only three Pkns have been demonstrated to be implicated in different aspects of *M. tuberculosis* infection. PksD is essential for *M. tuberculosis* brain endothelia invasion, and PknE participates in the anti-apoptotic mechanisms displayed by *M. tuberculosis* to avoid a host efficient immune response. Furthermore, PknE is involved in the resistance to nitrosative stress.³¹⁵ However, the in vivo role of PknG is less precise. The rest of the Pkns either seem to downregulate the in vivo replication (PknH, I, K and L), are dispensable for intracellular growth (PknF), or are essential for the basic metabolism, such as PknB and probably PknA.²⁸⁶

Proteases

Proteases play crucial roles in cellular homeostasis by controlling proteins involved in transcription, regulation, metabolism and virulence. Microbial pathogens frequently utilize extracellular proteases as virulence factors that can play different roles in tissue destruction or modulation of the immune response by inactivation of host defense molecules such as immunoglobulins and complement components. They can also activate key regulatory proteins or peptides, acquire nutrients by hydrolyzing host's proteins and process signaling molecules that regulate gene expression.¹³

The genome of *M. tuberculosis* H37Rv encodes over 100 proteases. Some are potential secreted proteins²² and 38 of them

are conserved among *M. leprae*, *M. bovis*, *M. avium paratuberculosis* and *M. tuberculosis*.³¹⁶ Despite this, very little is known about the biology of these enzymes in these organisms.

Serine proteases. Serine proteases are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the enzyme's active site. There is a family of five subtilisin-like serine proteases, the mycosins (mycosin-1 to -5), which share a high degree of similarity and are constitutively expressed in *M. tuberculosis* H37Rv. Bacterial subtilases are typically secreted and they degrade non-specific proteins to provide bacteria with readily importable peptides.³¹⁷

mycP1, *mycP3* and *mycP5* homologs have been detected in the genome of *M. leprae* and *mycP2* to 5 in *M. avium*. Only *mycP3* has been detected in the avirulent *M. smegmatis* so it appears that the multiplicity of the *mycP* genes may occur only in virulent mycobacteria.³¹⁸ *mycP1* encodes a protein that localizes to the cell wall/membrane fraction and is not expressed in the attenuated *M. bovis* strain BCG, although the gene is present in the genome. The expression of *mycP1* also occurs in intracellular bacteria and seems to be upregulated during growth in macrophages and may be processed intracellularly.³¹⁹

MycP1. Ohol et al. have recently reported that MycP1 controls ESX-1 protein export by cleaving EspB, one of the substrates of the ESX-1 secretion system.¹⁹¹ As it was mentioned in a previous section (see "Secretion systems"), ESX-1 secretion is required for early replication and full virulence in macrophages and mice.³²⁰ Other substrates of the ESX-1 system are ESAT6 and CFP10, both essential for virulence and highly immunogenic.³²¹⁻³²⁴ Thus, it may be essential for *M. tuberculosis* to tightly regulate the amount of ESAT6 being exported in order to maintain an optimal balance between virulence and immunogenicity. EspB is probably secreted as a full-length protein into the periplasm, promoting secretion of the ESX-1 substrates, but its proteolysis by MycP1 serves to turn off secretion. EspB is proteolyzed at least at three sites, two of which require MycP1, while the third one could be hydrolyzed by one of the four homologous of MycP1.¹⁹¹

The inability of $\Delta mycP1$ mutant bacteria to secrete ESAT6 leads to severe attenuation in the murine BMDM and BALB/c mouse model. While bacteria expressing a mutated version of MycP1 in the catalytic site grew to similar bacterial loads as wild type, they were attenuated in survival assays and histopathological analysis compared with wild-type *M. tuberculosis*. In this strain, the loss of MycP1 protease activity led to the constitutive secretion of ESAT6 and promoted ESAT6 antigen presentation by antigen-presenting cells during chronic infection, probably priming T cells to generate a stronger immune response against *M. tuberculosis*.¹⁹¹

HtrA. The HtrA (high-temperature requirement A) family of oligomeric serine proteases (S1, chymotrypsin family) is conserved from prokaryotes to humans. The structure of the protease comprises a serine protease domain and one or more C-terminal PDZ or protein-protein interaction domains, which regulate their protease activities by binding to regions of unfolded proteins in the periplasm. These proteins often possess the activities of chaperones and/or proteases.³²⁵ In bacteria, HtrA-like family members have been shown to participate in stress response

networks, including those regulated through sigma factors and two-component systems³²⁶ and they are involved in a variety of biological functions and pathogenicity.³²⁷

M. tuberculosis contains three HtrA proteases²² with a moderate homology among themselves (32–40% identity).³²⁸ HtrA1 (or DegP) has been predicted to be essential in *M. tuberculosis*.^{286,328,329} HtrA2 (or PepD) is involved in virulence,³²⁸ while HtrA3 (or PepA) is not (the $\Delta htrA3$ deletion had no effect on the mean survival time of mice or any other detectable phenotype).³²⁸ *M. tuberculosis* HtrA1 forms integral part of the membrane,³³⁰ while the other two proteases may be exported, as they are detected in the culture filtrate.^{329,331,332}

HtrA2 or PepD. HtrA2 (or PepD) encodes an HtrA-like serine protease and is thought to process proteins that have been altered following exposure of *M. tuberculosis* to extra-cytoplasmic stress. HtrA2 functions in vitro both as a protease and as a chaperone, and it is required for aspects of *M. tuberculosis* virulence in vivo. HtrA2 undergoes autolytic processing which might be involved in modulating its activity. C57BL/6 mice infected with a $\Delta htrA2$ *M. tuberculosis* mutant exhibit increased time to death, and less tissue pathology than animals infected with wild-type or complemented strains.³²⁸ However, loss of *htrA2* ($\Delta pepD$) neither alters the ability of *M. tuberculosis* to resist SDS-mediated killing nor it affects the growth and/or survival characteristics of *M. tuberculosis* either within peripheral human blood monocyte-derived macrophages or within resting or activated murine J774 macrophages in vitro. Rather, the deletion of *htrA2* in *M. tuberculosis* upregulates the expression of *sigE* and other stress-responsive determinants, which may compensate for the loss of this protein. In turn, the expression of *htrA2* is indirectly regulated by the extracytoplasmic function (ECF) sigma factor SigE³²⁶ and directly regulated by MprAB, the stress-responsive two-component signal transduction system encoded in operon *mprAB*. Remarkably, *htrA2* is part of this operon. Based on the similarities with the heat shock regulon CpxAR and RpoE systems in *E. coli*, White et al. have proposed a model for *M. tuberculosis*, in which MprAB and SigE systems sense and process stress resulting from the accumulation of unfolded or misfolded protein substrates and regulate the expression of chaperones/proteases like HtrA2. One identified target of HtrA2/PepD protease is the 35-kDa antigen of *M. tuberculosis* (Rv2744c), a member of the PspA family of proteins. These proteins participate in the phage shock response and likely in other multiple functions. The *Rv2744c* gene lies in an operon with *clgR*,³³³ a transcription factor that regulates its own expression and those of several other genes in *M. tuberculosis*, including proteases and chaperones.³³⁴ Therefore, one possible role of HtrA2 is to regulate the activity of proteins involved in maintaining cell wall homeostasis.³³³ *clgR* is further reviewed below in this section.

Rv3671c. Rv3671c is a membrane-associated protein,³³⁵ predicted to be a serine protease with conserved aspartate, histidine and serine active site residues and four transmembrane domains, two β -barrel subdomains and six antiparallel β -strands. It protects *M. tuberculosis* from acidification and oxidative stress probably by degrading proteins unfolded owing to acid and oxidative stresses. Alternately, it could be essential for

maintenance of the cell wall integrity, remodelling ion channels, proton pumps, or membrane lipids and thus, maintaining the internal pH.³³⁶

An *M. tuberculosis* transposon mutant disrupted in the *Rv3671c* gene, was sensitive to acid and failed to maintain intracellular pH homeostasis both in vitro and in activated BMDM. The mutant was also hypersensitive to the cell wall-damaging detergent SDS and to the lipophilic antibiotics erythromycin and rifampin, suggesting that it has some defect in cell wall function. Growth of the mutant was also severely attenuated in C57BL/6 mice and it induced markedly less pulmonary pathology than wild-type bacteria and complemented strains.³³⁵

ATP-dependent proteases. Self-compartmentalized or chambered proteases are common in most bacteria.³³⁷ *M. tuberculosis* encodes two ClpP (caseinolytic protease) homologs, ClpP1 and ClpP2, in a single operon, as well as the associated ATPase chaperones ClpC and ClpX (Rv2457c, Rv2667).³¹⁶ ClpP1 and ClpP2 are required for growth in vitro and in a mouse model of infection.³³⁸

The *clp* gene regulator (*clgR*) expression is highly increased in stress conditions such as heat shock³³⁹ and during macrophage infection.²¹⁵ ClgR activates the transcription of four protease systems (ClpP1/C, ClpP2/C, PtrB and HtrA-like protease Rv1043c) and three chaperones (Acr2, ClpB and the chaperonin Rv3269). ClgR-regulated transcriptional activation of these systems is essential for *M. tuberculosis* to replicate in murine BMDM. *M. tuberculosis* lacking ClgR is deficient in the ability to control phagosome pH. This attenuation could be explained if the rapid upregulation of the ClgR-dependent proteases and chaperones is necessary for protein homeostasis during exposure to the macrophage antimicrobial mechanisms, such as the oxidative burst. Regulation of proteases is vital to avoid the uncoordinated destruction of cellular proteins; this occurs at the level of substrate selection and also by controlling the expression of proteases via transcriptional regulators.³³⁴

Metalloproteases. Metalloproteases are a subfamily of proteases that use metals, mostly zinc, for their catalytic activities and are involved in virulence, cell wall processes and intermediary metabolism. There are three putative Zn-dependent metalloproteases in the genome of *M. tuberculosis*. We will only refer to two of them (Zmp1 and Rip1) because the third metalloprotease gene in *M. tuberculosis*, *Rv1977*, coding for a putative iminopeptidase that probably acts as a Zn-dependent enzyme with chaperone function, is deleted in *M. bovis*, so it is unlikely to be involved in virulence.

Zmp1. Master et al. have demonstrated that the *zmp1* (*Rv0198c*) gene is required for the survival of *M. tuberculosis* and BCG in murine J774 and RAW264.7 macrophages and for the full virulence of *M. tuberculosis* in a model of tuberculosis in C57BL/6 mice. It is also essential for prevention of inflammasome activation (a specialized inflammatory caspase activating protein complex, and component of the innate immune system) and IL-1 β production. The generation of active IL-1 β , a potent pro-inflammatory cytokine from its pro-protein precursor, is a tightly regulated process dependent on the inflammasome and caspase-1. Its production results in a vigorous host response to the pathogen and, when

induced, it is an effective anti-tuberculosis agent. *M. tuberculosis* inhibits this mechanism through the function of Zmp1.³⁴⁰

In a more recent study, Muttucumaru et al. have reported that Zmp1 is not required for survival of *M. tuberculosis* within THP1 macrophage-like human cell line, but the mutant in *zmp1* resulted hyper-virulent in SCID and C57BL/6 mice. The absence of Zmp1 led to changes in the expression of other genes in the same pathway or in compensatory pathways. Yet, the mechanism by which the deletion of *zmp1* leads to changes in gene expression is not known, but it is likely that the absence of this protease and its proteolytic activity results in the lack of the cleavage of a substrate signal for one regulatory protein, which in turn modulates the expression of other genes.³⁴¹ The discrepancies between these two studies are not well understood, but it could be due to the nature of the different *M. tuberculosis* mutants.

Rip1. Rip1 is a major virulence determinant of *M. tuberculosis* through its role in regulating cell envelope composition growth and persistence in vivo.³⁴² ECF sigma factors, alternative sigma factors that direct RNA polymerase to specific promoters (see "Sigma factors"), are often held inactive by trans-membrane anti-sigma factors, which are degraded by proteolysis in response to extracellular stimuli. Rip1 participates in signaling across the cell envelope through proteolysis of three anti-sigma factor substrates RskA, RslA and RsmA (anti-SigK, anti-SigL and anti-SigM, negative regulators of Sigma K, L and M respectively).³⁴³

Proteasome-associated proteins. The proteasome accessory or associated factors, Mpa and PafA, are important for defense against RNI and for virulence of *M. tuberculosis* in mouse. *mpa* (*Rv2115c*) encodes an ATPase with an AAA-protein family signature (AAA stands for ATPases associated with diverse cellular activities) homologous to that found in proteasome regulatory caps in eukaryotes³⁴⁴ and is probably involved in substrate recognition, unfolding and translocation into the proteasome core.^{345,346} In addition, the proximity of *Rv2115c* to putative proteosomal genes also indicates that this gene could be associated to proteosomal functions in *M. tuberculosis*.²² Darwin et al. have demonstrated that the products of *Rv2115c* (Mpa) and *Rv2097c* (PafA) confer *M. tuberculosis* protection against RNI, since mutants of *M. tuberculosis* H37Rv in any of these genes are highly susceptible to nitrite at low pH. *Rv2097c* encodes a hypothetical protein that was also potentially associated to proteosomal functions.³⁴⁵ The biochemical activity of PafA remains to be discovered, but it seems that PafA has a role similar to that of Mpa in protein degradation since *mpa* and *pafA* mutants were similarly sensitive to NO in vitro, and had the same attenuated phenotype in wild-type mice.³⁴⁷ When tested in iNOS^{-/-} mice, which do not produce NO in their macrophages, the *mpa* and *pafA* mutants were not as attenuated as in wild-type mice, supporting the hypothesis that these genes are required for resistance of *M. tuberculosis* to NO in vivo. However, both mutants not only showed impaired growth in resting primary macrophages from wild-type mice but also in those from iNOS^{-/-} mice, suggesting that *mpa* and *pafA* are involved in the response to more macrophage-associated stresses than those dependent on iNOS.³⁴⁵

As mentioned above, it has been hypothesized that both Mpa and PafA proteins are involved in proteasome functions to protect

the bacilli against nitrosative stress.³⁴⁵ It remains to be determined how the proteasome protects against NO and other stresses in the host. A plausible hypothesis is that Mpa, PafA and the proteasome dispose of NO-damaged proteins that are toxic to the cell. Another possibility is that the proteasome directly or indirectly regulates antioxidant or virulence gene expression.^{345,348} A role in bacterial growth both in vivo and in vitro has also been assigned to Mpa.³⁴⁹ An *M. tuberculosis* Erman mutant in *mpa* showed an impaired growth in standard culture medium and in BALB/c mice.³⁴⁹ Surprisingly, this gene showed to be not required for in vitro growth of *M. tuberculosis* H37Rv.³⁴⁵ Therefore, it is still a matter of discussion whether or not *mpa* can be considered a virulence gene of *M. tuberculosis* and what is the precise role of both, Mpa and PafA proteins in the mycobacterial proteasome.

Metal Transporter Proteins

Metals such as iron, magnesium, cobalt, copper, manganese and zinc are essential for survival of prokaryotes and eukaryotes either in the environment or within the cell. Metals are part of prosthetic groups or are co-factors of many enzymes. Microorganisms need traces of these micronutrients and in excess these metals could be toxic. Consequently, microorganisms have evolved many strategies in order to import metals into their cytoplasm or pump them out to the extracellular medium. Some of the proteins detailed in this section have shown to be essential for Mycobacterium growth in some in vitro extraordinary conditions, such as limiting metal condition. Although these proteins, according to our definition, are not actual virulence factors, they have been included in this review because they provide the bacteria with significant advantages for adaptation and survival in host cells.

Metal importers. *MbtB*, *IrtAB* and *IdeR*. MbtB, IrtAB and IdeR are proteins involved in iron acquisition. Iron is an essential cofactor, required in the heme of cytochromes and heme proteins. Iron is also cofactor of proteins involved in amino acid and pyrimidine biogenesis, enzymes involved in the tricarboxylic acid cycle and in DNA synthesis.³⁵⁰ In the restrictive environment of nature or of the mammalian macrophage, the iron is 1,000 times less concentrated than that required by the bacterium and it is also in an insoluble state. This situation has led pathogenic and non-pathogenic bacteria to evolve efficient iron-acquisition systems.

Siderophores are the most important iron-chelating compounds synthesised by microorganisms, being mycobactin and carboxymycobactin the major ones in Mycobacterium. These compounds are biosynthesized through the action of proteins encoded by the *mbt* cluster, which includes the genes *mbtA* to *mbtJ*. Proteins encoded by these genes are: MbtA, predicted as a salicyl-AMP lipase/salicyl-S-aryl carrier protein domain synthetase; MbtB, MbtE and MbtF predicted as non-ribosomal peptide synthetases; MbtC and MbtD predicted as polyketide synthases; MbtG a predicted lysine-N-oxygenase; MbtJ a putative esterase/ acetyl hydrolase; MbtI required for salicylic acid biosynthesis and MbtH of unknown function.³⁵¹ The mutation of any *mbt* gene disrupts the synthesis of these siderophores, which, in turn, unables the bacterium to acquire the metal from the medium. Therefore, the bacteria fail to survive in the host cell. As expected,

an *M. tuberculosis* H37Rv mutant, in which the *mbtB* gene was replaced by recombination with a hygromycin-resistance cassette, was restricted for growth in iron-limited media but grew normally in iron-replete media. In addition, the biosynthesis of all siderophores-like molecules derived from salicylic acid was interrupted in the mutant, a defect that impaired its growth in macrophage-like THP-1 cells as compared with the wild type.³⁵¹ These results clearly suggest that siderophore production is required for *M. tuberculosis* virulence.

An alternatively *mbt-2* cluster of genes involved in iron uptake is present in the *M. tuberculosis* genome. It includes two operons containing the *mbtK* to *mbtN* genes and the *irtA* and *irtB* genes, encoding for the IrtA and IrtB proteins which have the typical domains of an ABC transporter.³⁵² IrtA is different to the common ABC transporters; it has the transmembrane domain fused to a cytoplasm substrate-binding domain (SBD), which is essential for iron acquisition. IrtB only harbours the permease and ATPase domains.³⁵³ The inactivation of the *irtAB* system in *M. tuberculosis* H37Rv, by a two-step recombination, results in a mutant with a decrease ability to survive in iron-deficient conditions and shows reduced ability to use iron from the Fe-carboxymycobactin.³⁵⁴ These results indicate that IrtAB is a transporter of the Fe³⁺-siderophore complex. It has been proposed that IrtAB transports the Fe³⁺-siderophore complex toward the cytoplasm and, once there, the SBD domain of IrtA, functioning as a reductase, reduces the iron and releases it from the complex for its assimilation into metalloproteins.³⁵⁵ The relevance of IrtAB in the virulence of *M. tuberculosis* has been demonstrated in an *irtAB* mutant, which showed a reduced ability to replicate in THP-1 human macrophages and in the lungs of C57B/6 mice compared with the parental strain.³⁵⁴

The transcription of the *mbt* and *mbt-2* cluster of genes is negatively regulated by IdeR. IdeR is an iron-dependent regulatory protein essential in *M. tuberculosis* that functions as a repressor. In an abundant iron condition, IdeR is found complexed with Fe³⁺, and this complex binds to the promoter regions of *mbt* and *mbt-2* clusters preventing their transcription. On the contrary, in a depleted iron condition, there is not sufficient iron to form the Fe³⁺-IdeR complex, and IdeR releases the promoter leading to the transcription of the genes of this cluster which, as was described earlier, leads to the synthesis of proteins essential for the acquisition and incorporation of iron to the bacteria.³⁵⁶

MgtC. MgtC is a transmembrane P-type ATPase protein involved in Mg²⁺ uptake. Magnesium is essential as a cofactor for enzymes binding to phosphate and it is important in DNA and RNA synthesis. However, Mg²⁺ is found in low concentration inside macrophages. Therefore, the uptake of this metal is needed for the survival of the bacteria inside the host. The inactivation of *mgtC* in *M. tuberculosis* Erdman resulted in a mutant that grew slower than the parental or complemented strains in media with limiting magnesium (20 μM), but it grew equally to the parental strain in medium with high magnesium. In addition, in a medium containing limiting Mg²⁺ at slightly acidic medium (but not at neutral pH), the growth of the mutant was more affected than that of the parental or complemented strains, suggesting that in

the environment of the phagosome, that is, in a limiting and acidic environment, MgtC would be important in Mg²⁺ uptake. As expected, *mgtC* mutant was attenuated for virulence in cultured human macrophages and impaired for growth in the lungs and spleens of BALB/c mice compared with the parental or complemented strains.³⁵⁷ This suggests that the ability to acquire magnesium is essential for virulence in pathogens that proliferate within macrophage phagosomes.

Metal exporters. *CtpC.* CtpC is a Zn²⁺ efflux P-type ATPase that functions as an exporter. Even though Zn²⁺ is essential for bacterium growth, an increased concentration results poisonous to the bacteria. *M. tuberculosis* contains a set of 11 *ctp* genes, *ctpA* to *J* and *ctpV*, encoding for proteins predicted as probable cation transporters P-type ATPases, which unlike ABC transporters have auto-hydrolytic ATP activity required for exporting metals. It has been reported that macrophages may make use of heavy metal poisoning as mechanisms of antimicrobial immunity. Interestingly, a burst of free zinc inside macrophages and intraphagosomal zinc accumulation was observed a few hours post mycobacterium infection.³⁵⁸ In this condition of high Zn²⁺ concentrations, *M. tuberculosis* induces high level transcription of *ctpC* and the upstream *Rv3269* gene but also, at a lower level, of the *ctpG* and *ctpJ* genes, suggesting a role of CtpC in Zn²⁺ detoxification. In addition, an *M. tuberculosis* GC1237 mutant in *ctpC* gene, generated by allelic exchange, has shown higher levels (as much as three-times higher) of zinc retention within the mycobacterial cytoplasm compared with the wild-type strain. This mutant has also shown impaired intracellular growth in human macrophages.³⁵⁸ Taken together, these results suggest that the P1-type ATPases neutralize the toxic effects of zinc in macrophages by pumping the metal outside the mycobacteria.

CtpV. CtpV is a Cu²⁺ efflux transporter P-type ATPase required by *M. tuberculosis* to maintain resistance to copper toxicity. Copper is a required micronutrient but, similarly to Zn²⁺, is toxic at excess concentrations. A *ctpV* mutant, generated in *M. tuberculosis* H37Rv, has an increased copper sensitivity relative to wild-type or complemented strains when grown under toxic copper conditions (500 mM CuCl₂). Also, the mutant shows higher levels of intracellular copper than the wild type, suggesting that CtpV is necessary to maintain copper homeostasis.³⁵⁹ These authors have also demonstrated that CtpV has a role in host infection: when guinea pigs were infected with an *M. tuberculosis* H37Rv *ctpV* mutant, they showed significantly lower CFUs counts in lungs as compared with the wild-type or complemented strains. Additionally, the tissue damage and granulomatous responses were less severe in lungs infected with the *ctpV* mutant.³⁵⁹ These results suggest a connection between bacterial copper response mediated by CtpV and the virulence of *M. tuberculosis*, supporting the hypothesis that copper response could be important for intracellular pathogens.

It is clear that the metal transporter proteins, either importers or exporters, confer mycobacteria a significant adaptive advantage allowing them to survive in metal-limited environments, as inside the phagosome or the host, or to tolerate environment with high metal concentration that would otherwise be toxic and lead to bacterium death. That is why these proteins have been conserved

throughout the evolution from a saprophytic to an intracellular life into the host and why these proteins are relevant to the virulence of many bacteria. However, it is important to mention that based on the criteria here established to define a virulence factor, the proteins described in this section are not true virulence factors because their absence affect the in vitro growth of bacteria.

Gene Expression Regulators

M. tuberculosis is able to establish lifelong infections in individuals within granulomatous lesions that are formed following a productive immune response. Adaptation to this highly dynamic environment is thought to be mediated primarily through transcriptional reprogramming initiated in response to recognition of stimuli, including low-oxygen tension, nutrient depletion, reactive oxygen and nitrogen species, altered pH, toxic lipid moieties, cell wall/cell membrane-perturbing agents and other environmental cues.

To survive to the continued exposure to these potentially adverse factors, *M. tuberculosis* encodes a variety of regulatory factors, including 11 complete two-component systems (TCSs) and several orphan response regulators (RRs) and sensor kinases (SKs).

Two-component systems. Two-component regulatory signal transduction systems are important elements of the adaptive response of the tubercle bacillus, among other prokaryotes, to a variety of environmental stimuli. They typically consist of a membrane-bound histidine kinase (sensor kinase) that senses a specific environmental stimulus and a corresponding response regulator, phosphorylated by the sensor kinase that mediates the cellular response, mostly through differential expression of target genes (Fig. 2).

PhoP-PhoR. So far, 11 paired two-component systems have been described. Specifically, the two-component system PhoP-PhoR is the one whose disruption has been shown to most dramatically affect the ability of *M. tuberculosis* to replicate in cellular and animal models.

The *phoP-phoR* operon (conserved in most mycobacteria) encodes PhoR, a histidine protein kinase (sensor of stimuli), and PhoP, a transcriptional regulator that receives a phosphate from PhoR.

First thought to be implicated in phosphate metabolism and transport, the two-component system PhoP-PhoR is involved in diverse aspects of metabolic physiology, lipid metabolism regulation and respiration. PhoP-PhoR is likely to sense magnesium. It increases triacylated mannose-capped lipoarabinomannans (ManLAM) acyl forms. Monoacylated ManLAM, which predominates in *phoP-phoR* mutants, fails to inhibit the IL-12 production in human dendritic cells. Many genes involved in lipid metabolism seem to be regulated by PhoP-PhoR: *pks3* (polyketide β -ketoacyl synthase), *pks5* (polyketide synthase), *papA3* (polyketide synthase associated protein), *fadD26* (a fatty acid-coenzyme A ligase), *lipF* (an esterase/lipase), *fbpA* (secreting type Ag85A FbpA), *mmpL10* and *mmpL8* (transport proteins belonging to the RND superfamily). Some *M. tuberculosis* lipid components such as DAT, PAT and SL are also diminished in the *phoP* mutants.³⁶⁰

In the attenuated *M. tuberculosis* H37Ra strain, a single nucleotide mutation within a DNA binding domain of PhoP can abolish the binding between PhoP and its own promoter.³⁶¹ *M. tuberculosis* H37Rv Δ *phoP* shares many features with *M. tuberculosis* H37Ra, such as low content of DAT, PAT and SL, and show significant overlap in their transcription expression profile. This is strong evidence of the critical role played by PhoP-PhoR in metabolic control with a considerable impact on the virulence of *M. tuberculosis*. Moreover, PhoP regulates genes related to ESX-1 secretion system required for virulence and ESAT6 secretion. PhoP modulates the expression of both EspB (secreted ESX-1 substrate protein B) and EspR (transcriptional regulatory protein).³⁶² As a result, *M. tuberculosis* H37Rv Δ *phoP* and *M. tuberculosis* H37Ra synthesize, but are incapable of secreting, both ESAT6 and CFP10.

Importantly, *phoP* and *phoP-phoR* mutants of *M. tuberculosis* are attenuated for growth in various cultured or primary cell types, including murine BMDM, murine alveolar macrophages, murine J774 macrophage cells, human THP-1 cells, and SCID and BALB/c mice and guinea pigs. These ex vivo and in vivo assessments of the role of PhoP-PhoR to *M. tuberculosis* virulence have been reviewed by Bretl et al.³⁶⁵

Abramovitch et al. have suggested that a specific subset of the *phoP* regulon is controlled by *aprABC* operon,³⁶⁶ since similar altered expression profiles were detected in *M. tuberculosis* mutants for either *phoP-phoR* or *aprABC* locus. The authors have demonstrated that the *aprABC* operon, which is unique to the MTBC, is expressed in acidic medium in vitro and in macrophages in a manner dependent on PhoP-PhoR. Moreover, this study showed that the deletion of *aprABC* operon from *M. tuberculosis* CDC1551 caused defects in intracellular replication in both resting and activated C57BL/6 BMDM, but also affected the mycobacterial in vitro growth, showing the mutant aggregation in liquid media and reduced colony size.³⁶⁶ These authors propose a model where PhoP-PhoR senses the acidic pH of the phagosome and induces *aprABC* expression to fine-tune processes unique for intracellular adaptation of *M. tuberculosis*.

SenX3-RegX3. This TCS is involved in phosphate sensing and is homologous to the master aerobic regulator ArcB-ArcA of *E. coli*.

In *M. tuberculosis*, RegX3 both positively and negatively regulates a large and functionally diverse regulon comprised of 100 genes. Several of these genes encode hypothetical proteins, while others are involved in important physiological activities, including energy metabolism, cell envelope maintenance, and regulatory functions.³⁶⁷

RegX3 is an activator of the *phoA* gene that encodes the alkaline phosphatase, PhoA. In turn, PhoA activates the expression of *ptsS* belonging to the phosphate transport system PstSCAB. RegX3 also upregulates the expression of another phosphate transport system, PhnDCE. Phosphate limitation restricts *M. tuberculosis* growth in a concentration-dependent manner. Three other genes, *ald*, encoding alanine dehydrogenase, *cyd*, encoding a subunit of the cytochrome D ubiquinol oxidase, and *gltA1*, encoding a citrate synthase, also are regulated by SenX3-RegX3 system.³⁶⁸

In the intergenic region of *senX3* and *regx3* genes, there is a mycobacterial interspersed repetitive unit (MIRU) element,

precisely the first one to be identified.³⁶⁹ While *regX3* appears to be essential in *M. smegmatis*, this gene is dispensable for the in vitro growth of *M. tuberculosis*.³⁶⁷

senX3 and *regX3* are necessary for *M. tuberculosis* full virulence,³⁶⁷ as a *M. tuberculosis* H37Rv *senX3-regX3* mutant is attenuated for growth in the human THP-1 macrophage cell line and murine BMDM (I15), similar to what is observed in the lungs and spleens of SCID and DBA mice after infection with a this mutant.³⁶⁷ Also, individual *senX3* and *regX3* mutants were attenuated in BALB/c mice.³⁷⁰

DosR/S/T (DevR/S/T). Together with PhoP-PhoR, DosR/S/T is the most studied TCS system from the MTBC. This TCS system is formed by one response regulator (DosR), which is activated in response to hypoxia and nitric oxide, and by either DosS or DosT, both histidine kinases. This TCS was initially termed DevR/S/T by the original discoverers. DosR refers to “dormancy survival regulator” and this denomination has been at present adopted by most researchers. Boon and Dick have observed that DosR is responsible for the dormancy stage of *M. tuberculosis*, and that upon mutation of DosR the bacteria failed to enter in dormancy and died in a Wayne culture system of hypoxia.³⁷¹ It has been observed that exposure of *M. tuberculosis* mutants lacking DosR to low oxygen tension induces the expression of more than 100 genes, from which 48 are under the control of DosR whose C-terminal segment recognizes a 18–20 bp palindromic sequence upstream of the regulated genes.³⁷² Both DosS and DosT autophosphorylate and transfer the phosphate group to DosR. DosS and DosT are indispensable for the induction of the Dos regulon in a dormancy model in vitro. DosS is expressed during hypoxia and DosT is constitutively expressed. However, once expressed DosS can be replaced by DosT. DosS and DosT possess GAF domains, a receptor of cyclic GMP and a β -type heme group that senses CO and NO molecules,³⁷³ which are a cell signal of hypoxia and a marker of bactericide activity by macrophages respectively. Heme oxygenase-1 produces carbon monoxide in the macrophage, inducing the *M. tuberculosis* Dos regulon.

DosT is inactive when bound to O₂. However, during hypoxia, CO and NO may displace O₂ in DosT, restoring its active form. O₂ can oxidize the heme group and inhibit DosS. The oxidative form Fe³⁺-DosS does not autophosphorylate, while the reduce Fe²⁺-DosS does.³⁷³ On the other hand, in hypoxia, FAD and FMN reduce the heme group from DosS further supporting the concept that DosS is a redox sensor.

The emerging and highly pathogenic *M. tuberculosis* Beijing strain normally overexpresses the Dos regulon during the exponential growth phase, and many isolates of Beijing lineage possess a frameshift mutation in DosT. The molecular explanation for this difference has yet to be found.³⁷⁴

In spite of the central role of the DosR/T/S system in *M. tuberculosis* sensing of oxygen tension and redox state, its involvement in virulence is not clear, yielding contrasting results. For example, the persistence of an *M. tuberculosis* H37Rv mutant in Δ *dosR* seemed to be unaltered in C57BL/6 mice; this mutant, however, produced less lung pathology.³⁷⁵ Other authors have shown that in C57BL/6 and BALB/c mice, rabbit and guinea pig models, a Δ *dosR-S* mutant exhibited a slight growth defect and

induced lung pathology generation when compared with the wild-type *M. tuberculosis*.^{376,377} Malhotra et al. have observed that guinea pigs infected with a *devR* mutant showed a significant decrease in gross lesions in lung, liver and spleen; and a three log lower bacterial burden in spleen compared with guinea pigs infected with the parental strain.³⁷⁸ Contrary to this, a *devR* deleted of *M. tuberculosis* showed hypervirulence in an SCID mouse model. Δ *devR* also grew more rapidly in the acute stage of infection in immunocompetent DBA mice.³⁷⁹

MprA/MprB. Named after mycobacterial persistence regulator, MprB is the sensor kinase and MprA is the response regulator in this two-component system. MprB not only phosphorylates MprA but also acts as its phosphatase, regulating in this way its own activity.³⁸⁰

The genes coding for this TCS are part of an in vivo-expressed genomic island in which a set of 20 genes are activated only during BALB/c mice infection and not in SCID mice or in vitro. The region is called an in vivo-expressed genomic island (iVEGI).³⁸¹

The use of MprA and B deletion mutants has led to the identification of around 200 genes that are regulated by the pair. Positive regulation is observed in *mprA*, *pepD* and *moaB2*, as well as in *acr2 (hspX)* (a member of the DosR regulon).³⁸²⁻³⁸⁴ This TCS is part of a highly complex regulatory network and, as a consequence, it is difficult to define the *mprAB* regulon. For example, *sigE* and *sigB* are regulated by MprAB system that responds to membrane damaging and stressing agents such as detergents and alkaline pH. SigE regulates *ppk1* that generates polyphosphates, which are in turn phosphate donors for MprB. Also, MprA and SigE regulate *pepD* that cleaves Rv2774 protein, implicated in cell wall antibiotic susceptibility. Mutants in *mprA* or in both *mprAB* genes are hypervirulent in human macrophages derived from peripheral blood monocytes but attenuated in C57BL/6 mice,³⁸⁵ indicating that this complex regulatory system is required for full virulence. Moreover, *mprAB* orthologous genes are required for *Rhodococcus equi* virulence.³⁸⁶

The authors of the whole genome sequence of *M. tuberculosis*²² have noted that it encodes for 11 putative complete TCS and a few isolated kinase and regulatory genes. This number is clearly lower than in *B. subtilis* and *E. coli*, whose genomes encode more than 30 genes for two-component regulatory systems.²² Some of these TCS were not included in this review because mutants in these systems have not been constructed or obtained. Among them, there is *Rv0600c-Rv0601c-trcA*,³⁸⁷ which is composed of two SKs (*Rv0600c* and *Rv0601c*) and one RR, but the signals sensed by the SKs have not yet been identified. Similarly, mutants in MtrA-MtrB have not been obtained, although it has been described that the overexpression of *mtrA* causes attenuation in mice.³⁶⁵ Some others may be essential for *M. tuberculosis* growth, as no mutants could be obtained. In our knowledge, no articles using alternative gene inactivation approaches such as conditional lethal or gene silencing have been published in order to solve the question of the role of these TCS in virulence. In other cases, the mutation of a TCS has no a clear impact in virulence. One example is *narL-Rv0845*, encoding a TCS similar to NarQ-NarL from *E. coli* that regulates genes expression in response to nitrate

concentrations. The deletion of genes encoding this TCS produced no alteration of *M. tuberculosis* virulence in mice.³⁷⁹ The mutation of TCS *prpA-prpB* diminishes the growth in macrophages only at initial stages and has no impact on mice infection.³⁶⁵ Other mutants like those of *kdpD-kdpE* and *trcX* (*Rv3765c*)-*trcY* (*Rv3764c*) have been tested only in SCID mice. Moreover, mutation in *trcR* (*Rv1033c*)-*trcS* (*Rv1032c*) has produced a hypervirulence effect.

PhoP/PhoR and DosR/T/S are the most studied TCSs in *M. tuberculosis*. Even though PhoP-PhoR have been studied extensively regarding its role in the virulence of *M. tuberculosis*, the input signals and the molecular aspects of sensing need more research. For example, it has been postulated that PhoP senses Mg²⁺,¹⁹⁰ but other authors failed to reproduce this finding (C. Martin, University of Zaragoza, Spain, personal communication). Regarding the role of DosRTS in virulence, the results are inconclusive or even contradictory. Despite the current understanding of the signals sensed, a central respiratory gas, such as CO₂, has not been tested as a heme ligand for these sensors.

Sigma factors. Bacteria adapt to changes in the environment and life-style mainly using RNA polymerase (RNAP) holoenzymes with different promoter specificities. The holoenzyme comprises a core RNAP with five subunits and a dissociable subunit called sigma factor (σ). Sigma factors contain many of the promoter recognition determinants that provide promoter specificity to the RNAP holoenzyme. Therefore, the association of a variety of sigma factors with the core RNAP allows the transcription of genes required for the different environmental conditions that the bacteria encounter during its cycle of life.

M. tuberculosis encodes 13 sigma factors (σ A, σ B, σ C, σ D, σ E, σ F, σ G, σ H, σ I, σ J, σ K, σ L and σ M), of which σ A is the main sigma factor and the other are alternative sigma factors. Along with the alternative sigma factors, ten (σ C, σ D, σ E, σ G, σ H, σ I, σ J, σ K, σ L and σ M) are members of the so-called ECF subfamily, comprising sigma factors that respond to signals from the extra-cytoplasmic environment.³⁸⁸

This section summarizes current knowledge regarding mycobacterial sigma factors that have been demonstrated to be essential for the full virulence of *M. tuberculosis*.

σ A (*SigA*). SigA, also known as RpoV,³⁸⁹ is the main sigma factor that regulates housekeeping genes and its expression may be induced in response to stress, but increasing evidence indicates that SigA also regulates the expression of virulence genes.^{389,390} The first evidence of this role was the finding that attenuation of a defined *M. bovis* mutant strain, assessed in a guinea pig model, was restored when a wild-type copy of *sigA* genes was introduced in this strain by electroporation. Further analysis of the *sigA* sequence of the *M. bovis* attenuated strain has shown that this mutant has a single point mutation at position 522, which caused an arginine \rightarrow histidine change.³⁸⁹ The mutation is located in a region highly conserved among major sigma factors and their homologs, with the characteristic helix-turn-helix motif known as to probably interact with promoters.³⁹¹ However, the same *M. bovis* *sigA* mutant did not show attenuation in Australian brushtail possum,³⁹² supporting the idea that mycobacteria employ different mechanisms to replicate and survive in its hosts.

In *M. tuberculosis*, overexpression of *sigA* improves the bacterial replication in mononuclear phagocytes and in C57BL/6 mice compared with control strain.³⁹⁰ Knockdown of *sigA* in two *M. tuberculosis* strains negatively affected the bacterial replication inside a human macrophage cell line and in lungs of C57BL/6 mice, and elicited a minimal local inflammatory response.³⁹⁰ Moreover, it has been reported that SigA interacts with WhiB3, a putative transcription regulator and that the inactivation of *whiB3* conferred an attenuated phenotype to a wild-type *M. bovis*, thus, suggesting that the SigA-WhiB3 interaction was the cause of the observed attenuation.³⁹³ However, inactivation of *whiB3* in *M. tuberculosis* resulted in partial attenuation of its virulence,³⁹³ implying that the genetic background may define the relevance of particular genes in the virulence of pathogenic mycobacteria.

σ C (*SigC*). SigC is important for pathogenesis and survival within granulomas in low-dose aerosol guinea pig infection model: *sigC*-mutant produced fewer and smaller lung and spleen granulomas as compared with the parental *M. tuberculosis* H37Rv.³⁹⁴ In an *M. tuberculosis* CDC1551 background, the mutation of *sigC* significantly impaired the replication of the bacilli in lungs of infected guinea pigs,³⁹⁴ and it dramatically reduced its lethality in DBA2 and SCID mice.^{394,395} However, there are reported discrepancies in the impact of *sigC* in the bacterial replication in immunocompetent DBA2 mice organs. While one study³⁹⁵ showed that the mutant replicates at the same rate as the parental strain in mice organs, another one³⁹⁴ reported that the infection remained between 1 to 1.5 log¹⁰ units lower than the replication of *sigC*-mutant three weeks post infection. In the latter study, equivalent rates of replication were detected in lung of SCID mice infected with the wild-type and mutant strains.³⁹⁴ Although the role of *sigC* in the replication of *M. tuberculosis* in mice needs more investigation, the role of *sigC* in the immunopathology induced by *M. tuberculosis* in animal models, in terms of both mortality and histopathologic progression of pulmonary disease, is undoubted.

In this regards, it has been shown that lack of *sigC* produces reduced levels of pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IFN- γ in the lungs of both SIDC and DBA2 mice and reduced level of neutrophils, suggesting that the attenuated phenotype of the *sigC* mutant is associated with its inability to trigger a strong early immune response. Consistently, an *M. tuberculosis* H37Rv mutant in *sigC* did not produced necrotic granulomas in lungs and spleen of guinea pigs.³⁹⁶

A complete genomic microarray study has demonstrated that SigC modulates the expression of several key virulence-associated factors including a crystallin homolog *hspX*, a two-component sensor kinase *senX3* and a two-component response regulator *mtrAr*.³⁹⁵

Altogether, these results indicate that SigC is an important regulator of virulence. Additionally, *sigC* together with *sigE* are the only ECF sigma factors encoded by the reduced *M. leprae* genome, therefore, supporting the role of *sigC* in the virulence of pathogenic mycobacteria.²⁸⁴ Moreover, *sigC* is only present in the genome of pathogenic mycobacteria.

σ D (*SigD*). The role of SigD in the virulence of *M. tuberculosis* has been evaluated in H37Rv and CDC1551. Mutants of *sigD* in

both *M. tuberculosis* strains are able to replicate and persist in the lungs and spleen of immunocompetent mice at similar rates as those of the parental strain. However, the survival of mice infected with either of the two mutant strains is moderately improved as compared with that of the parental strain. In addition, no such longer survival is detected in immunocompromised CB17-SIDC mice infected with the mutant strain.³⁹⁷ Immunopathology associated to *sigD* is found in lungs of BALB/c mice,³⁹⁸ but not in those of C3H mice.³⁹⁷

In regards to the genes whose expression is regulated by SigD, two independent global transcriptional studies have defined different subsets of SigD-targeted genes. While the analysis of Raman et al. has identified a gene encoding the autocrine growth factor RpfC and a gene of unknown function, *Rv1815*, as directly being regulated by this sigma factor during exponential growth phase, Calamita et al. have reported that SigD governs the expression of a small set of ribosomal genes expressed in stationary phase during in vitro growth. In conclusion, evidence obtained so far indicates that *sigD* may play a role in the tuberculosis disease, but further investigation is needed to clearly establish the action of SigD in regards to the mechanism of virulence of *M. tuberculosis*, for instance: which genes are regulated by SigD, and in which in vivo situation these genes are expressed.

σE (*SigE*). The ECF σE is one of the major regulators involved in the mycobacterial stress responses^{326,399,400} and it is also upregulated upon macrophage infection.^{229,400,401} Moreover, the stress-responsive two-component system MprAB directly regulates the expression of *sigE*, as well as the expression of *sigB*, another stress-responsive sigma factor gene in *M. tuberculosis*.³⁸²

A *sigE* mutant strain of *M. tuberculosis* has been independently constructed and characterized by two laboratories in the CDC1551 and H37Rv strains.^{402,403} The disruption of *sigE* in both strains resulted in delayed lethality when the strains were used to infect both immunocompetent and immunocompromised mice. However, while the replication of the mutant in H37Rv was impaired in BALB/c mouse lungs, as compared with its parental strain,⁴⁰³ this attenuated phenotype was not detected in C3H/HeJ mice infected with the CDC1551 mutant strain.⁴⁰² This apparent discrepancy on the ability of *sigE* mutant to replicate and persist in mouse lungs could be explained in part by the differences in the genomic background of both the bacterial and mice strains used in these studies. In addition, the mutation of *sigE* impaired the growth of H37Rv in both human and mice macrophages⁴⁰⁴ and in human dendritic cells,⁴⁰⁵ suggesting that this sigma factor controls genes directly related to the intracellular survival of the bacterium. These findings are also consistent with the proposed role of *sigE* in the response of *M. tuberculosis* to oxidative stress.⁴⁰⁴

Most of the genes regulated by σE are involved in the maintenance of *M. tuberculosis* cell envelope integrity and lipid metabolism.⁴⁰⁶ Importantly, it has been recently reported that σE is a global regulator of the central metabolism genes of the methylcitrate cycle,⁴⁰⁷ a metabolic pathway for the assimilation of propionyl-CoA produced during catabolism of lipids.⁴⁰⁸ Because *M. tuberculosis* activates the lipid catabolism during the host infection, the involvement of *sigE* in this metabolic pathway also supports its role in the mycobacterial virulence. Moreover, the

global transcriptional profile of *M. tuberculosis* infected macrophages has demonstrated that σE induces the expression of host genes involved in pro-inflammatory response,⁴⁰⁶ which could explain the alteration in the granuloma structure observed in mouse lungs infected with an *M. tuberculosis* mutant in *sigE*.⁴⁰⁹

σF (*SigF*). It has been shown that σF is upregulated upon nutrient depletion of *M. tuberculosis* cultures⁴¹⁰ and during infection of cultured human macrophages.⁴⁰¹ Based on these and other evidence,⁴¹¹ σF has been defined as a stress response alternative sigma factor of *Mycobacterium* species. However, the viability of a mutant of *sigF* in *M. tuberculosis* CDC1551 strain was unaltered either under prolonged nutrient starvation conditions or upon macrophage infection,⁴¹² questioning the role of σF in the responses to stress conditions, at least in this mycobacterial species.

Although the mutant of *sigF* (mentioned above) produced a lethal infection of mice, it was less virulent than its parental strain in a time to death analysis.⁴¹³ In a long-term virulence assay the mutant strain replicated in the lung and spleen of immunocompetent mice at moderate lesser rates than the parental strain.⁴¹⁴ Moreover, at a later stage of the disease, the mutant in the CDC1551 strain showed smaller and fewer lesions and less inflammation in the lungs and spleen.⁴¹⁴ This view is consistent with another study in which a *sigF* mutant of *M. tuberculosis* H37Rv produced diffused granulomas lacking necrosis in guinea pig lungs.³⁹⁶

σG (*SigG*). *sigG* is significantly upregulated within human macrophages.^{229,415} It has been proposed that SigG is also involved in the SOS response of *M. tuberculosis*,⁴¹⁶ but recent evidence suggests the opposite.⁴¹⁷ Consistently with an essential role of *sigG* during intracellular growth of *M. tuberculosis*, an *M. tuberculosis* deleted in *sigG* gene displayed impaired survival in a macrophage infection model.⁴¹⁶ Despite these findings, the relevance of *sigG* for the infection of *M. tuberculosis* in animal models remains to be demonstrated.

σH (*SigH*). σH is a key regulator of the response to oxidative, nitrosative, and heat stresses in *M. tuberculosis* and other mycobacterial species.⁴¹⁸⁻⁴²⁰ Microarray analysis has shown that σH regulates the expression of other sigma factors, several heat shock proteins, detoxification enzymes, virulence factors and protein processing, among others proteins.⁴²¹ This sigma factor is significantly upregulated in human macrophages⁴⁰¹ and in its absence *M. tuberculosis* showed impaired replication in monkey macrophages at late stage.⁴²² In this last macrophage model, a mutant *M. tuberculosis* strain in the *sigH* gene induced the expression of numerous inducible and homeostatic β -chemokines and several apoptotic markers, suggesting a role of σH as a modulator of innate immune responses directed against *M. tuberculosis*.⁴²² Moreover, an *M. tuberculosis* mutant deleted in *sigH* did not produce acute tuberculosis when tested in a non-human primate model of acute tuberculosis.⁴²³ In resistant C57BL/6 mice an *M. tuberculosis* CDC1551 *sigH* mutant showed significantly reduced lethality, comparing to the wild-type strain, but high bacterial counts in lung and spleen. In addition, the mutant produced less tissue pathology in lungs than the parental strain. In susceptible C3H mice, the mutant again showed

diminished immunopathology, and the survival of mice infected with the mutant was significantly recovered as compared with that of animal infected with the wild-type strain.⁴²⁴ Altogether, these findings suggest that σ H modulates the immune response elicited against *M. tuberculosis*.

σ L (SigL). σ L regulates the expression of proteins involved in lipid metabolism and cell envelope, such as polyketide synthase, lipid transporters, enzymes of lipid biogenesis, etc., among other mycobacterial genes.^{425,426}

The relevance of *sigL* in *M. tuberculosis* virulence has been addressed in two independent studies. Hahn et al. have shown that a *sigL* mutant of *M. tuberculosis* produced less lethality in BALB/c mice than its parental strain.⁴²⁶ On the other hand, Dainese et al. have performed a similar analysis using two different mouse strains: the resistant C57BL/6 and the susceptible DBA/2 mice strains. In these two animal models, the mutant strain replicated and persisted in organs at a similar rate to that of the parental strain. Consistent with the finding of Hahn et al., a significant extension of the survival time was reported for DBA/2 mice infected with the *sigL* mutant strain, comparing to that of mice infected with the parental strain.⁴²⁵ Therefore, the lack of *sigL* in *M. tuberculosis* results in an immunopathology defect (*Imp* phenotype) of virulence in mice as it has been reported for mutants in many other sigma factors.

While sigma factors B, I, J and M have shown so far to be dispensable for in vivo growth of MTBC species, mutants in the sigma factors A, C, D, E, F, G, H, K and L were attenuated at least in one infection model, showing differences in their attenuated virulence phenotypes. When tested in either mice or guinea pigs, mutants in sigma factors F, H, L and likely D have revealed an *Imp* phenotype, in which high tissue bacterial counts were observed but the tissue pathology and lethality were reduced. σ C and σ E are, to our knowledge, the only examples of a sigma factor with a role in the immunopathology of tuberculosis as well as bacterial replication in mouse organs. However, it is important to take in consideration that attenuated virulence phenotypes are sometime restricted to the animal model used. For instance, it has been recently reported that a mutant in *sigH* was not only severely attenuated for lethality and immunopathology but also for bacterial burden, when assayed in non-human primates.⁴²³ Little information about the role of σ K and σ G in the virulence of pathogenic mycobacteria is still available. While a mutant in *sigG* has only been addressed in a macrophage infection model, the unique evidence of σ K as a virulence factor was obtained from a signature-tagged mutagenesis experiment; in which *sigK* gene showed to be part of a locus lost in an attenuated *M. bovis* strain.⁴²⁷

As expected, in MTBC species, virulence sigma factors regulate the expression of genes essential for a successful infection. For instance, σ C regulates the expression of several key virulence-associated genes, such as *hspX*, *senX3* and *mtrA*³⁹⁵; σ D controls the expression of the *Rv0169*, a member of the virulence *mce1* operon³⁹⁷; σ E governs the expression of *esat-6*, *sodA* *fbpB* (*ag85B*), *fbpC* and *icl*;⁴⁰⁴ σ F is involved in the regulation of biosynthesis and structure cell envelope and lipid metabolism, among other processes;⁴¹⁴ the mutation of *sigG* in *M. tuberculosis*

have been demonstrated to affect the expression of *sigH* and *sigD*, showing the complex interplay of sigma factors in *M. tuberculosis*. Similarly, under diamine stress, σ H regulates the expression of several sigma factors, together with many virulence genes with a wide variety of functions, such as: *mce1* operon, *pirG*, regulatory virulence genes, *cfp10* and *sodA*, among others.⁴¹³ Finally, the regulation of the virulence genes *pks10* and *pks7*, both involved in fatty acid metabolism, by σ L, has been demonstrated in two independent studies.^{425,426} In addition, it has been reported that the in vivo dispensable sigma factors M and J govern the expression of four *esat-6* homologs and alternative H₂O₂ resistance pathway, respectively.^{428,429} Moreover, transcriptional analysis of an *M. tuberculosis sigB* mutant strain revealed regulation of the virulence gene *katG*, among others.⁴³⁰ Altogether, this evidence suggest that almost all of the sigma factors control the expression of potential virulence genes of MTBC, irrespectively their role in the bacterial virulence.

It is remarkable that, with the exception of σ C, sigma factors are conserved in non-pathogenic mycobacterial species, suggesting that the regulatory systems are conserved across the Mycobacterium genus, whereas the regulon under their control varies across species.

Other transcriptional regulators. The genome of *M. tuberculosis* encodes more than one hundred putative transcriptional regulators (<http://genolist.pasteur.fr/TubercuList/>). This extensive platform of regulatory genes together with those encoding two-component systems and sigma factors would indicate that there is a fine-tuning at the level of protein expression defining the success of pathogenic mycobacteria in infection, colonization and persistence inside of hosts. The regulatory genes are classified in families: AraC, TetR, MarR, GntR, LuxR, AcrR, ArsR, LysR, AsnC and CRP/FNR among others, based on the presence of conserved domains; and depending on their effects in the transcription, they are repressors, activators or both. Members of all these subfamilies are present in the *M. tuberculosis* genome, but, surprisingly, few have been mutated; thus, the impact of these mutations in general physiology and virulence needs further studying.

Some regulatory genes have been mutated in pathogenic mycobacteria, such as *mce3R*,¹⁰² *araC*,⁴³¹ *mabR*,^{102,432} *furB* and *furA*^{433,434} and a subset of them have been assessed in infection models. For instance, it has been demonstrated that the lack of Mce1R in *M. tuberculosis* increases the virulence of the bacteria in a mice model,^{435,436} likely due to overexpression of *mce1* operon. Other regulatory mutants, most of them mutants in activators, are attenuated either in animal models, macrophages or both. Below, we describe the main features of this kind of attenuated regulatory mutants.

Rv0485. *Rv0485* is a highly conserved gene in mycobacteria and other closely related species, which encodes a putative transcriptional regulator and a member of the NagC/XylR repressor family.^{22,437} The disruption of *Rv0485* in *M. tuberculosis* leads to a reduced expression of the *pe13* (*Rv1195*), *ppe18* (*Rv1196*) and *Rv2626c*, as well as overexpression of a putative operon *Rv2391* to *Rv2394*. Members of the PE and PPE gene families seems to be involved in different aspects of mycobacterial

pathogenesis, for example, in granuloma and macrophage persistence⁴³⁸ acid resistance, vacuole acidification⁴³⁹ and induction of apoptosis and pro-inflammatory cytokine secretion.⁴⁴⁰ The *Rv0485* mutant strain replicates in organs of both immunocompetent and immunocompromise (SCID) BALB/c mice at a similar rate as the wild type, but with milder lung pathology, and the survival of animals inoculated with the mutant is modestly longer than that of those inoculated with the wild type. These findings suggest that disruption of *Rv0485* alters the immunomodulatory characteristics of the mutant strain rather than impacting on the growth or strain clearance.⁴³⁷ Since the mutation alters the expression profile of *M. tuberculosis* in both senses (up- and down-regulations) and the relationship among these *Rv0485*-regulated genes is not obvious, it is difficult to speculate about the role of this regulon in the virulence of *M. tuberculosis*; thus, more research is necessary to clarify the function of *Rv0485*.

Rv1931c. *Rv1931c* encodes a putative AraC transcriptional regulator. A region of *Rv1931c* gene has been deleted from *M. tuberculosis* strain 1424 genome and the resultant mutant has been tested in mice and in macrophages. The mutant strain exhibited reduced replication in mouse BMDM compared with the parental and complemented strains.⁴⁴¹ The replication of the mutant was also impaired in lungs and spleen of mice, but the attenuated phenotype of the mutant was more consistent during chronic infection. Although there is no available information on the genes regulated by *Rv1931c*, altogether, these results suggest that this protein is directly or indirectly implicated in the expression of virulence factors in *M. tuberculosis*.

HspR. *hspR* (*Rv0353*) encodes the transcriptional repressor HspR, a member of the MerR family. In the *M. tuberculosis* genome, *hspR* is the fourth gene in an operon also comprising *hsp70*, *grpE* and *dnaJ*, all of them encoding heat shock proteins.

It has been experimentally demonstrated, by proteomic analysis, that HspR represses the expression of Hsp70, ClpB, GrpE and DnaJ by binding to a consensus upstream sequence known as HAIR (HspR-associated inverted repeats).⁴⁴² The transcriptional analysis of an *M. tuberculosis* *hspR* deletion strain indicated that HspR controls also the transcription of *Rv0251c-Rv0249c* operon, which encodes Acr2, a member of the low-molecular-mass α -crystallin family.³³⁹ However, it has been later reported that the transcription of *acr2* is also regulated by MprA.³⁸³ Importantly, the transcriptional regulation exerted by HspR resulted relevant for the persistence of *M. tuberculosis* in mice. The *M. tuberculosis* mutant in *hspR* showed reduced survival and less histological damage in C57BL/6 mouse organs than the parental strain during the chronic phase of infection. However, no complementation studies have been performed to confirm the role of HspR in these observed attenuated phenotypes. On the other hand, when assayed in BMDM, the mutant was as virulent as the wild-type strain. The authors have proposed an immune-mediated attenuation on the *hspR* mutant owing to overexpression of Hsp70. This is supported with their finding that an *M. bovis* BCG mutant in *hspR* induced higher production of IFN γ in splenocytes of infected mice as compared with its parental strain.²¹⁴ Therefore, overexpression of Hsp70, and very likely of the other HspR-regulated proteins, seems to favor the host immune response

against pathogenic mycobacteria. This hypothesis is consistent with the antigenic properties of heat-shock proteins.^{443,444}

WhiB3. WhiB3 is one of the seven members of the WhiB-like regulator family of *M. tuberculosis*. The role of WhiB3 in the virulence of pathogenic *Mycobacterium* species has been extensively addressed. Steyn et al. have found that this regulator binds to the main sigma factor of *M. tuberculosis*, SigA,³⁹³ suggesting a concerted transcriptional control between both transcriptional regulators. Singh et al. have reported that WhiB3 senses redox signals through its four Fe-S cluster.⁴⁴⁵ The same authors further suggested that WhiB3 responds to the reductive stress generated by host lipid catabolism by controlling the expression of *pks2*, *pks3* and *fbpA* genes, which are involved in the synthesis of the methyl-branched lipids PAT, DAT, SL-1 and TMM/TDM.⁴⁴⁶ Consistently, an *M. tuberculosis* mutant in *whiB3* showed alteration in the production of these methyl-branched lipids, as well as PDIMs and triacylglycerol (TAG), both in vitro under defined oxidizing and reducing condition and inside macrophages. Therefore, the authors have proposed that under intracellular oxidative or reductive stress, *M. tuberculosis* modulates the anabolism of diverse polyketides to maintain the redox homeostasis. WhiB3 would participate in this process by channeling toxic reducing equivalents, produced as a result of host lipid catabolism, into bacterial lipid anabolism. The absence of *whiB3* in *M. tuberculosis* induced the production of pro- and anti-inflammatory cytokines by macrophages, without modifying its intracellular replication. It is most probable that this modulation of macrophage innate immune response is due to the lack of WhiB3-mediated regulation of complex lipids that *M. tuberculosis* uses to suppress the host immune responses. Therefore, it is tempting to speculate that WhiB3 contributes to the virulence of pathogenic mycobacteria by indirect modulation of the host immune response.⁴⁴⁶

Remarkably, the mutation of *whiB3* in *M. bovis* significantly impaired the bacterial replication in spleen of guinea pigs, and this attenuated phenotype is reverted in the complemented strain.³⁹³ Taking into consideration that some methyl-branched lipids are not produced by *M. bovis*, it is plausible that the lipid regulation mediated by WhiB3 has a more essential role in *M. bovis* for modulating the host immune response in its favor.

In another study, Banaiee et al. have demonstrated that the lack of WhiB3 in *M. tuberculosis* H37Rv did not alter the replication of the bacteria in C57BL/6 mouse or guinea pig organs, but extended the survival of infected immunocompetent mice as compared with the wild-type strain.⁴⁴⁷ However, the time to death curve of immunodeficient IFN- γ R^{-/-} mice infected with an *M. tuberculosis* *whiB3* mutant was equivalent to that of mice infected with the parental strain. Although these results appear to be contradictory, it is important to take into consideration the differential immunological features of the two models used in these studies.

MosR (*Rv0348*). MosR (named derived from regulator of mycobacterial operons of survival) regulates the transcription of several operons and regulons, either by repression or activation, including those involved in mammalian cell entry (*mce1*), hypoxia (*tgs1*) and starvation. In fact, by means of global transcriptional

examination, it has been found that MosR indirectly regulates the transcription of 163 genes. Consistent with the high number of MosR-regulated genes, the analysis of *M. tuberculosis* transcriptional regulatory network indicates that MosR is the most connected hub of this network.⁴⁴⁸

Disruption of the *mosR* gene from the genome of *M. tuberculosis* H37Rv impairs the replication of the bacteria in lungs of BALB/c mice, but this attenuated phenotype is more evident after 30 weeks post-infection, suggesting a critical role for *mosR* in the chronic phase of tuberculosis. In addition, the mutant strain is less lethal to BALB/c mice as compared with the wild-type strain. The reintroduction of a wild-type copy of *mosR* into the mutant produces a partial complementation in vivo.⁴⁴⁹ Although the heterologous expression of *M. tuberculosis mosR* gene in *M. smegmatis* would indicate that MosR participates in anaerobic responses,⁴⁴⁹ the precise role of this global regulator in the virulence of pathogenic mycobacteria is still unclear.

VirS. *VirS* of *M. tuberculosis* belongs to the AraC family of transcriptional regulators (AraC/Xyls) and controls the transcription of the *mymA* operon, which is encoded divergently to *virS*.⁴⁵⁰ In turn, *VirS* is phosphorylated by the serine/threonine kinase PknK, and this phosphorylation increases the affinity for the *mymA* promoter.²⁹⁸ *M. tuberculosis* mutants in either *virS* or *mymA* operon exhibited reduced contents and altered composition of mycolic acids together with the accumulation of saturated fatty acids as compared with the parental strain. These mutants were more susceptible to major anti-tubercular drugs at acidic pH and also showed increased sensitivity to detergent and to acidic stress than the parental strain.

The disruption of *virS* resulted in an impaired replication of *M. tuberculosis* in spleen of guinea pigs at chronic phase of infection, but not in lungs as compared with the complemented and wild-type strains. Moreover, this reduced virulence for *virS* mutant strain was also detected upon infection of activated J774 murine macrophage cell line but not in resting macrophages, indicating a role of *VirS* in the response to oxidative burst. Importantly, the mutant in the *mymA* operon showed the same attenuated phenotype observed for *virS* mutant.³² The role of the *mymA* operon in the intracellular replication and persistence of *M. tuberculosis* has been further studied in many in vitro models. For instance, an *M. tuberculosis* mutant deleted in *mymA* operon failed to grow in human THP-1 cells, pneumocyte cells (A549) and murine J774 macrophage cells⁴⁵¹ as compared with the complemented and wild-type strains.

Altogether, these results indicate that the expression of the *mymA* operon, activated by *VirS*, is required for maintaining the appropriate mycolic acid composition and bacterial wall permeability. Each of these postulated biologic roles is consistent with the attenuation observed for *virS* and *mymA* mutants.

PhoY2. *phoY2* encodes a probable phosphate-transport system transcriptional regulatory protein. However, the role of *PhoY2* as a transcriptional regulator has not been assessed yet. It has been demonstrated that its *E. coli* homolog, *PhoU*, is a global repressor for cellular metabolism involved in the generation of persistent bacteria.⁴⁵² Remarkably, the disruption of *phoY2* in *M. tuberculosis* H37Rv increased its susceptibility to rifampicin and

pyrazinamide. Moreover, the mutant was less capable to survive and persist in mouse lungs and spleens than the complemented and parental strains.⁴⁵³ These observed phenotypes for the *phoY2* mutant suggest that *PhoY2* may have a role in the generation of persistent *M. tuberculosis*. However, further studies are needed for deciphering the function of this putative regulator, as well as for identifying its target genes.

Proteins of Unknown Function

The advent of the genomic era has allowed sequencing the genome of 12 species of Mycobacterium. This, added to the studies in the transcriptomic, proteomic, metabolomic and bioinformatic fields have allowed to know or to infer the biological function of many ORFs. Despite this, the physiological function of many proteins related to Mycobacterium virulence remains unknown.

Below, we describe MTBC proteins of unknown function, in which their involvement in bacterial virulence has been demonstrated.

The PE/PPE families. The PE/PPE families have been found only in mycobacteria and consist of two large unrelated families of acidic and glycine-rich proteins whose genes are clustered.²² About 10% of the coding capacity of *M. tuberculosis* H37Rv genome consists of *pe* and *ppe* genes. Many of these genes are located upstream or within the *ESX* operons. The PE name derives from the signature motif Pro-Glu, at the residues 8 and 9 on the N-terminus, whereas the PPE represents the Pro-Pro-Glu motif. Most of these proteins are localized on the cell surface and/or are secreted, and they induce a strong immune response in the host. The N-terminus of these proteins is well conserved and is linked to a variable C-terminus, which is thought to be a source of antigenic and genetic variations.⁴⁵⁴⁻⁴⁵⁶ The high level of polymorphism among members of these protein families suggests a role in the mechanism of host immune-response evasion, even though a wide variety of functions have also been assigned to them.⁴⁵⁷ However, whether or not these PE/PPE proteins modulate the host's immune system in favor of the bacilli is still uncertain, since only few PE/PPE proteins have shown to be certainly relevant for the virulence of MTBC. Some of these virulence associated-PE/PPE proteins are encoded in the *ESX-1* locus, which is detailed in the section on Secretion Systems; the PE_PGRS30 protein, which belongs to the PE family is detailed in "Phagosome arresting" and the rest are described below.

PE_PGRS33 is a cell surface protein encoded by the *Rv1818c* gene that promotes mycobacterial aggregation.^{458,459} This aggregative phenotype was observed in a BCG mutant for the *Rv1818c* gene, which was unable to form typical large cell aggregates when grown in liquid media but rather showed a dispersed growth phenotype, suggesting a role of this protein in cell surface structure. The role of this protein in mycobacteria virulence was studied in a macrophage model. Murine J774 macrophage cells were infected with the BCG mutant and its replication was significantly reduced as compared with the parental and complemented strains.¹⁹⁴ Similarly, PE_PGRS51, encoded by *Rv3367*, and PPE46, encoded by *Rv3018*, have shown to be

essential for full replication of *M. tuberculosis* mutants in mouse organs. *M. tuberculosis* H37Rv mutant in the *Rv3367* gene replicated less than its wild-type strain in lung and spleen of C57BL/6J mice¹⁰⁰ and an *M. tuberculosis* strain MT103 mutant in *Rv3018* have a reduced multiplication rate in the lung of BALB/c mice compared with wild type.⁴² Although the precise mechanism by which these virulence associated PE/PPE proteins are involved in the survival and replication of MTBC in their host is still uncertain. The findings here described highlight the role of these exclusive proteins in the virulence of mycobacteria.

Other proteins with unknown functions. By means of TraSH technique, it has been demonstrated that *Rv1099c* and *Rv0573c*, two proteins predicted as conserved hypothetical proteins (CHP), are required for *M. tuberculosis* survival during infection in a mouse model of tuberculosis.¹⁰⁰ Individual mutants in each gene were constructed and used to infect C57BL/6J mice. In fact, mutant growth in lung and spleen did indeed show a significant defect compared with that of the parental strain, indicating that these proteins have a role in virulence but that their mechanisms are still unknown.¹⁰⁰

The *Rv0204c* and *Rv2452c* proteins are predicted as an integral membrane protein and a hypothetical protein, respectively. None of them show any sequence similarity to entries in the protein database. Mutant strains in both genes generated in *M. tuberculosis* strain MT103 background have shown reduced multiplication in the lung of BALB/c mice compared with wild type.⁴²

The *Rv1290c*, *Rv1891* and *Rv3404c* proteins are described as CHP. CB-17/Icr SCID mice infected with *M. tuberculosis* H37Rv mutants for *Rv1290c*, *Rv1891* or *Rv3404c* presented a highly significant increase in their survival time as compared with mice infected with the parental strain.⁴⁶⁰ For instance, the survival time of the mice infected with the mutant in the *Rv1290c* gene increased from a median of 25 to 62 d.⁴⁶⁰ This strong in vivo attenuation for this mutant clearly shows that *Rv1290c* has an important function in determining the extent of the virulence of *M. tuberculosis*.

The *Rv1503c* to *Rv1506c* proteins, encoded by the *Rv1503c* to *Rv1506c* operon, are also predicted as CHP. *M. tuberculosis* transposon mutants in the *Rv1503c* or *Rv1506c* genes fail to transcribe the entire *Rv1503c* to *Rv1506c* operon. These mutants grew poorly inside host macrophages and their ability to infect BALB/c mice was strongly impaired, since the CFU counts from spleen and lungs were markedly reduced as compared with the parental. The attenuated phenotype was restored to wild-type levels in a complemented strain carrying the entire operon.⁴⁶¹ Interestingly, the absence of the *Rv1503c* to *Rv1506c* proteins in the mutants lead to a noticeable overproduction of tetracylated sulphoglycolipids (Ac₄SGL), and to lower production of 2,3-di-O-acetylrethalses as compared with the parental or complemented strains.⁴⁶¹ This imbalance could indicate that these proteins play a role in glycolipid metabolism and this may account, at least in part, for the attenuated phenotype observed in mutants.

Rv0199 is a probable conserved membrane protein with homology to some Mce-associated proteins.¹⁰¹ It has been predicted to have a transmembrane domain at the N-terminus,

the majority of which is located on the extra-cytoplasmic side of the membrane.¹¹⁹ The *Rv0199* gene is located in the virulence-associated membrane proteins (VAMP) region, together with 10 genes that encode for VAMP.¹⁰¹ While the function of *Rv0199* is still unknown, its role in the mycobacteria virulence has been already demonstrated in a TraSH analysis performed in a mouse model of infection.¹⁰⁰ Furthermore, an *M. tuberculosis* mutant by a transposon insertion in the *Rv0199* gene was defective for growth in macrophages as compared with the wild type, and this intracellular growth defect was complemented by addition of a plasmid expressing *Rv0199*.¹¹⁹

MmpL4 is predicted as a probable conserved transmembrane transport protein. It belongs to the MmpL family of proteins (see "Lipid and Fatty Acid Metabolism") but, unlike them, a role in fatty acid transport has not been yet reported. An *M. tuberculosis* H37Rv mutant in *mmpL4* used to infect C57BL/6 mice was significantly impaired for growth as compared with the parental strain.⁴⁵ The mutant strain is so attenuated that the same phenotype was observed in a more susceptible mice model of tuberculosis, a (C57BL/6xDBA2) F₁ hybrid mouse race. Although the mutation in the *mmpL4* strain has not been complemented, the expression of the genes around *mmpL4* has been evaluated by an RT-PCR experiment demonstrating that the insertion of the *hyg* cassette did not cause a polar effect.⁴⁵ Thus, the attenuated phenotype is likely due to the absence of *MmpL4*.

Finally, *Rv2136c* is predicted as a possible conserved transmembrane protein of unknown function with a possible role in the virulence of *M. tuberculosis*. An *M. tuberculosis* transposon mutant in *Rv2136c* was hypersensitive to acid pH, SDS, heat shock, reactive oxygen and nitrogen intermediates and to lipophilic (rifampicin or ethambutol) or non-lipophilic (isoniazid) antibiotics.¹¹⁴ The mutant was unable to maintain a neutral cytoplasmic pH in activated macrophages and its growth and survival in C57BL/6 mice was severely attenuated in lungs and spleen as compared with the wild type.¹¹⁴ These results suggest an important role for this protein in the virulence of the bacteria. However, the lack of complementation of the mutant phenotype with either the single *Rv2136c* gene or the putative operon (*Rv2133* to *Rv2137c*) could indicate a possible alternative mutation as the cause of the defects observed.

In conclusion, just over half (52%) of the open reading frames in the *M. tuberculosis* genome have been assigned a biological function, while the rest of the ORFs are annotated as hypothetical proteins of unknown function.⁴⁶² The PE/PPE family of proteins are the most relevant in this group; despite that their specific function remains unknown, they have been linked with the virulence of mycobacteria as a source for antigenic biodiversity and as modulators of the host immune response.^{454,456} However, almost half of the ORFs in the mycobacteria genome remain as uncharacterized proteins, and most of these proteins have no homology with other and in many cases are unique and play a specific role in the organism. Therefore, our understanding on the pathogenicity and virulence of mycobacteria is greatly reduced. Moreover, in the current era of informatics, the in silico analyses are essential and the use of tools such as BLAST, PFAM, COG, among others, have allowed us to predict the function of proteins

described as yet hypothetical based on the degree of homology they share with those whose function have been determined by biochemical and/or molecular studies. Therefore, it has been possible to predict the function of 12% of the hypothetical proteins in the *M. tuberculosis* genome, with a confidence greater than 75%.^{463,464} Our challenge now is to integrate the large amount of data resulting from the sequencing of genomes with the annotation of biological function for each ORF and, thus, be able to build the puzzle which involves the understanding of the biology of the organisms in order to have the proper artillery to control the development of pathogenic bacteria.

Other Virulence Proteins

Regions of differences (RD). Only RD1 and RD2 have been demonstrated to be essential for the full virulence of the MTBC species among the already known region of differences. RD1 has been already described in the previous section.

RD2 is absent in some BCG sub-strains. The deletion in BCG goes from *Rv1978* to *Rv1988* and comprises 12 genes. In *M. pinnipedi* there is a deletion of 1.94 kb involving *Rv1978* and *Rv1979c*. In addition, some *M. microtti* strains have a deletion going from *yrb3A* (*Rv1964*) to *Rv1979c*.⁴⁶⁵ Little is known about the biological function of the proteins encoded in this region apart from the fact that many of the proteins encoded here have been shown to be antigenic. A putative amino acid efflux pump gene, *lysE* (*Rv1986*), from *M. tuberculosis*, together with the divergently transcribed putative *lysR*-type regulator gene (*Rv1985c*), is encoded in RD2.⁴⁶⁶ *Rv1987* is a putative cellulose-targeting protein. *Rv1982c* is predicted to encode a toxin-antitoxin of the VapBC family; this family of proteins has been shown to inhibit translation through RNase activity.¹⁸⁵ RD2 was disrupted in *M. tuberculosis* H37Rv to test whether its loss might contribute to the attenuation of BCG.⁴⁶⁷ The deletion of RD2 did not affect in vitro growth; in contrast, the mutant manifested a decrease in pulmonary and splenic bacterial burdens and reduced pathology in C57BL/6 mice at early time points. This attenuated phenotype was complemented by reintroducing the genes *Rv1979c* to *Rv1982* (including *mpt64*), but not *Rv1985c* to *Rv1986*. *Rv1983* and *Rv1984* have been naturally deleted in a clinical strain of *M. tuberculosis*, demonstrating that these genes are dispensable for full virulence in humans.⁴⁶⁸ In RAW264.7 macrophages, the mutant H37Rv Δ RD2 has shown a decreased proliferation and impaired modulation of the host innate immune response; both observed phenotypes were complemented with *Rv1979c* to *Rv1982* genes.⁴⁶⁷

The RD2-containing BCG Russia, BCG Pasteur (which has a natural deletion of RD2) and a BCG Pasteur strain complemented with RD2 genes *Rv1979c*-*Rv1982* have been compared through various in vitro and in vivo assays for immunogenicity and protection. In a mouse vaccine-challenge model, the presence of RD2 displayed no effect on pulmonary TB, as measured by *M. tuberculosis* burden and degree of histopathology, until 12 weeks post-infection. RD2 deletion was, however, associated with decreased dissemination of *M. tuberculosis* to the spleen. The data demonstrate that the loss of RD2 resulted in decreased immunogenicity but did not affect protection against pulmonary

TB, indicating dissociation between these phenotypes commonly associated with BCG vaccination.⁴⁶⁹ *Rv1980* encodes for Mpt64, a well-known antigen and exported protein containing an N-terminal signal sequence whose function is presently unknown. Surprisingly, no attempts to mutate *mpt64* gene to assess the impact on virulence have been published so far.

Acg. In *M. tuberculosis*, *acg* transcription is controlled by the TCS DosR-DosS in response to hypoxia and nitric oxide conditions within macrophages and mice.⁴⁷⁰ *Acg* had been first proposed as a member of a superfamily of classical nitroreductases.⁴⁷¹ However, Hu et al. have shown that the deletion of *acg* from *M. tuberculosis* does not affect bacterium growth and survival in acidic, nitric oxide and hydrogen peroxide environments in vitro.⁴⁷² Besides, this mutant was attenuated in macrophages and in acute and persistent murine infection models.⁴⁷² The replication of the *acg* mutant strain was significantly impaired in both resting and activated BMDM. In addition, BALB/c mice infected with the mutant strain showed significant lower bacillary load in organs and longer survival than its parental strain and less cytokine production in lungs (TNF- α , IL6 and IL-1b at later times). Although the authors of this study have not addressed the impact of the *acg* mutation in the in vitro culture of *M. tuberculosis*, it is plausible to speculate that this mutation has only affected the in vivo growth of the bacilli, since it has been possible to obtain the mutant in standard conditions in vitro growth.

PckA. *pckA* encodes a phosphoenolpyruvate carboxykinase that catalyzes the reversible decarboxylation and phosphorylation of oxaloacetate (OAA) to form phosphoenolpyruvate (PEP) or the reverse reaction. It has been demonstrated that the expression of *pckA* is upregulated in the presence of either palmitate or acetate. The *pckA* disruption in *M. bovis* BCG results in attenuation of the obtained mutant strain in macrophages and mice. The mutant strain replicates less in both spleen of BALBc mice and resting murine BMDM than its parental strain.⁴⁷³ The authors have postulated that the attenuation observed shortly after infection with the mutant in *pckA* is due to a reduced capacity to respond to the changing environment within the macrophage, possibly when OAA is needed to fulfil an anaplerotic role in the tricarboxylic acid cycle.

PtpB. Reversible phosphorylation of proteins by protein kinases and phosphatases is a major mechanism of signal transduction events that regulate several cellular processes. The *M. tuberculosis* genome encodes two tyrosine phosphatase, *ptpA* (*Rv2234*) and *ptpB* (*Rv0153c*),⁴⁷⁴ but, surprisingly, it apparently does not encode any tyrosine kinase. Both tyrosine phosphatases have been shown to be important for the virulence of *M. tuberculosis*. It has been reported that PtpA is relevant for the arrestment of phagosomal maturation by *M. tuberculosis* (see "Phagosome arresting"), and thus, to survival inside macrophages. However, this phosphatase seems to be nonessential for bacterial in vivo replication. On the other hand, an *M. tuberculosis* mutant in *ptpB* was less persistent in guinea pig spleen than wild-type and complemented strains. Moreover, animals infected with this mutant showed a reduction in the percentage of granuloma in liver and lung and a highly lymphocytic granuloma when compared with the wild-type and complemented strains. The authors of this study have suggested that the histological

phenotype observed in guinea pigs infected with the *ptpB* mutant is consistent with an infection controlled by the host immune response. The mutant has also displayed a moderated attenuation in IFN γ activated murine J774 macrophages,⁴⁷⁵ but not in resting macrophages. These findings highlight the role of tyrosine phosphatase in the pathogenesis of *M. tuberculosis*.

Hsp22.5. The study of Talaat et al. describes an *M. tuberculosis* genomic region containing about 20 genes highly expressed in BALB/c mice. Among these, there is an in vivo upregulated gene, *Rv0990c*.³⁸¹ As the expression of *Rv0990c* has been also shown to be upregulated at high temperature³³⁹ and under treatment with H₂O₂ and SDS, this protein (named as Hsp22.5) has been proposed as a novel heat shock protein. The deletion of *Rv0990c* from *M. tuberculosis* H37Rv genome affects the replication of the bacteria in lungs of BALB/c mice during chronic phase of the disease. However, this attenuation is not detected in acute infection of guinea pigs. In addition, the survival of mice infected with the mutant strains is longer than that of the wild type. The introduction of a wild-type copy of *Rv0990c* in the mutant strain partially complements the lethal phenotype of *M. tuberculosis*.⁴⁷⁶ These results, together with the findings that the mutation of *hspX* and *acr2* genes results in alteration of the *M. tuberculosis* virulence, highlight the role of heat shock proteins during the infection of pathogenic mycobacteria. Although the function of Hsp22.5 is still unclear, expression profile studies have shown that the absence of this protein in *M. tuberculosis* affects the expression of a large number of genes, among them several heat shock proteins.⁴⁷⁶

Concluding Remarks

Global searches have allowed the identification of more than a hundred potential virulence genes in pathogenic mycobacteria. A pioneering work in this field was that of Camacho et al., who used STM to identify *M. tuberculosis* mutants exhibiting an attenuated phenotype in large pools of mutants.⁴² Remarkably, most of the genes identified in that study have been implicated in lipid metabolism. Later on, the outstanding work of Sasseti and Rubin¹⁰⁰ allowed the definition of 194 genes as specifically required for mycobacterial growth in mice by using the TraSH methodology. Again, lipid metabolic genes as well as those involved in the transport or metabolism of inorganic ions and carbohydrates were prominently represented among the genes required for in vivo growth. Although data from these high-throughput screenings have

been considered in this review, the main focus is mainly on those genes whose role in virulence has been individually demonstrated. Remarkable, more than 20% of these loci have also been identified as virulence factors using the global virulence analysis of Sasseti and Rubin.¹⁰⁰

The virulence factors described in this review are mainly involved in the interaction of MTBC species with the host macrophages: One set of these virulence factors is implicated in the adaptation of the bacilli to the limited nutritional condition of the macrophages and includes proteins required for the uptake of nutrients and ions as well as for the switching of carbon metabolism that occurs when mycobacteria reside inside host cells. Another set comprises proteins that participate in the mechanisms triggered by mycobacteria to counteract the microbicidal host cell responses, such as: (1) arresting the normal progression of the phagosome and increasing the resistance to host toxic compounds (cell wall barrier and specific effectors), (2) escaping from the intracellular compartment and (3) avoiding the development of localized, productive immune responses.

An additional category of virulence factors is that encompassing proteins with a role in the modulation of host immune responses. Among them, those implicated in the inhibition of inflammatory responses and apoptosis are highly represented. Mutant strains in genes encoding these virulence proteins have been shown to induce poor tissue damage and, in some cases, wild type levels of bacteria replication in organs.

The thorough description of *M. tuberculosis* virulence factors summarized in this review is expected to contribute to a better understanding of the mechanisms involved in the interaction of pathogenic mycobacteria with their hosts. This information is, thus, essential for the development of new treatments and vaccines that should help prevent or control this pandemic. However, it is important to take into consideration the words of Smith, "TB will be completely eradicated only when poverty and unequal development are ended throughout the world."⁷

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