Cellular signaling pathways and transcriptional regulation in *Mycobacterium tuberculosis*: Stress control and virulence

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***Mycobacterium tuberculosis* is a successful pathogen that overcomes the numerous challenges presented by the immune system of the host. This bacterium usually establishes a chronic infection in the lung where it may silently persist inside a granuloma until a failure in host defenses can lead to the reactivation of the disease. An increasing number of molecular genetic tools available in the past few years have made possible the study of signal transduction and transcriptional regulatory mechanisms that contribute to the ability of *M. tuberculosis* to be so well adapted.**

This review intends to summarize the current knowledge about the major molecular components involved in signaling pathways and in transcriptional regulation in *M. tuberculosis* with specific emphasis on their relation to bacterial pathogenesis and the ability to cope with stresses inside the host.

MYCOBACTERIUM TUBERCULOSIS is the causative agent of tuberculosis (TB). This disease together with HIV and malaria is one of the main causes of mortality due to an infectious disease[1]. Two billon people are infected in the world, 10% of them will develop TB at some point in their lives and 2 million die each year from the disease[2]. Although TB has been the focus of medical research for more than 100 years, *M. bovis* BCG, the only vaccine developed and used for the last 70 years is not completely satisfactory due to great variation in its efficacy[3]. Currently used front-line antibiotics can be effective but these are not available in all places in the world, and there is also the severe problem of newly emerging drug-resistant strains due to the use of inferior drugs or noncompliance[4]. Infection of a mammalian host by *M. tuberculosis* occurs primarily by the aerosol route. The lung is usually the principal organ affected, and the bacteria initially reside in alveolar macrophages[5]. In those hosts that cannot control the bacterial infection, *M. tuberculosis* can overcome the hostile conditions and is usually able to replicate in the macrophages. To survive in this cell type, *M. tuberculosis* has developed strategies to arrest phagosome maturation at an early stage, maintaining a relative non-acidic pH and avoiding fusion with lysosomes[6]. An immuno-competent individual infected with *M. tuberculosis* is usually able to develop a strong immune response whereby numerous peripheral blood monocytes and T lymphocytes migrate to the lung and contain the focus of infection by forming a granuloma. In these stages of containment the bacteria may remain for several months or years usually until the person becomes immuno-compromised for some reason (e.g. immunosuppressive drugs, AIDS, aging, etc.). This state of dormancy and persistence is one of most intriguing phenomena of *M. tuberculosis* lung infection.

To be so well adapted to the numerous environmental conditions that the host offers, *M. tuberculosis* must be able to mount an array of functions, like inducing metabolic pathways to utilize the carbon source available inside the macrophage, scavenging oxygen radicals to avoid cellular damage, and acquiring iron, to name a few. Thus, to establish a successful infection, bacteria have to constantly sense the medium, and efficiently signal the changes that enable a quick adjustment to new conditions. This series of events occurs coordinately by means of numerous mechanisms of signal transduction and transcriptional regulation. Signal transduction in bacteria mainly involves the so-called two component systems, however mycobacteria also have several serine/threonine kinases and tyrosine phosphatases that were originally thought to participate only in eukaryotic signal transduction. After the signals indicating environmental changes are sensed and transduced, bacteria respond by synthesizing new proteins and down regulating others. This balanced regulation of gene expression in bacteria occurs primarily at the level of transcription, largely through the activity of DNA-binding proteins called repressors or activators. As discussed below, the coordinate operation of the molecular machinery involved in bacterial signaling and transcription is essential for *M. tuberculosis* to successfully adapt inside the host.

Two-component systems

The two-component systems form a large family of proteins involved in signal transduction that allow bacteria to detect and respond to many different kinds of stimuli. This signaling mechanism is widespread throughout the prokaryote world and is also found in some eukaryotes. The basic two-component system consists of two proteins, a sensor and a receiver, that are involved in a

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phosphotransfer reaction. After interaction with the appropriate stimulating ligand, the sensor protein, called a histidine kinase, can bind and hydrolyse ATP, catalysing the autophosphorylation of a conserved histidine residue founded in all histidine kinases and producing a high-energy phosphoryl group[7–9]. The phosphate is then transferred to the associated receiver protein, known as response regulator, at a conserved aspartic acid residue generating a high-energy acyl phosphate[10,11]. Once the phosphotransfer occurs, the response regulator is activated, allowing it then to carry out its specific function[12–14]. In most cases, the response is the modulation of transcription though the phosphorylated response regulator interacting with DNA at specific binding sites located in target gene promoter regions. The total effect is changes in global gene expression that aid the organism in responding to the initial signal sensed by the histidine kinase. Most histidine kinases and response regulator are highly conserved, however, there is little sequence similarity in the sensing domain of different histidine kinases. This variety of different extracellular, intracellular and/or transmembrane sensor domains accounts for the different types of molecules that can initiate the signal.

Two-component systems are involved in the signal transfer from initial stimulus to cellular response for a multitude of different processes. Some examples include: pathogenesis and virulence (*Salmonella typhimurium* PhoP–PhoQ)[15,16], chemotaxis (*Escherichia coli* CheA–CheY/CheB)[17], pilus production (*Pseudomonas aeruginosa* PilS–PilR)[18], adhesion (*P. aeruginosa* FleS–FleR)[19], osmoregulation (*E. coli* EnvZ–OmpR)[20,21], phosphate sensing (*E. coli* PhoR–PhoB)[22–25] and sporulation (*Bacillus subtilis* KinA–KinB–Spo0F–Spo0B–Spo0A)[26–29].

The published genome of *M. tuberculosis* H37Rv reveals the presence of eleven paired two-component systems, five unpaired response regulators, and two unpaired histidine kinases[30]. With the idea of identifying systems involved in virulence, a preliminary step in the characterization of the *M. tuberculosis* two-component systems involves mutagenesis of the individual systems and testing mutants for survival in macrophages and mice. Presently, many laboratories are mutating the *M. tuberculosis* two-component systems and performing such experiments. Of the eleven-paired systems in the *M. tuberculosis* genome, nine have been transcriptionally disrupted in either the response regulator or the histidine kinase and the resulting mutants' growth phenotype have been tested *in vivo*. In many cases these mutants have not been complemented, then the presence of suppressor mutations arising during the original mutagenesis, have not been ruled out.

M. tuberculosis response regulator transcriptional regulation in macrophages

In an attempt to identify which two-component systems are induced during a macrophage infection, and hence possibly required for virulence, Deretic *et al.* characterized the gene expression of *M. tuberculosis* response regulators in 7H9 medium (*in vitro*) and in macrophages (*ex vivo*). Using green fluorescent protein (GFP) expression vectors fused with the promoters of select *M. tuberculosis* response regulators[31], constructs placed in *M. bovis* BCG and *M. tuberculosis* were screened for GFP expression during infection of macrophages. They found the response regulators *phoP* and *Rv0818* to be constitutively expressed *in vitro* and *ex vivo* in *M. bovis* and *M. tuberculosis*. The response regulator, *mtrA*, was induced in both *M. bovis* and *M. tuberculosis* in macrophages, while the response regulators *Rv0981* and *Rv3143* were induced in macrophages in *M. bovis* but not in *M. tuberculosis*. The following response regulators showed no induction in macrophages: *narL*, *Rv0903c*, *Rv1033c*, *Rv3765c*, *Rv1626*, and *Rv2884* (ref. 31). Since *mtrA* was the only response regulator induced during macrophage infection but not in broth culture, these workers attempted to delete the *mtrA–mtrB* system, but were unsuccessful, suggesting that this system is essential for growth[32]. While this work was an initial step in understanding the response regulators role in *M. tuberculosis* pathogenesis, a lack of gene induction in macrophages does not rule out their involvement in virulence since there are many aspects to *M. tuberculosis* infection and disease progression. Also, constitutively active response regulators may still be important for *M. tuberculosis* survival in macrophages, as seems to be the case with the PhoP–PhoR two-component system.

PhoP–PhoR (Rv0757–Rv0758)

The role of two-component systems in the ability of a pathogen to successfully mount an infection has been well documented with the most extensively studied system involved in bacterial pathogenesis being the *Salmonella enterica serovar typhimurium* (*S. typhimurium*) PhoP–PhoQ system[15,16,33]. Knockout strains of either the response regulator PhoP or the histidine kinase PhoQ cause *S. typhimurium* to become avirulent[15,16]. The function of the PhoP–PhoQ two-component system is to detect the concentration of Mg^{2+} and to a lesser extent calcium (Ca^{2+}) that is available to the bacterial cell[34]. In a low Mg^{2+} environment, the PhoP–PhoQ system upregulates transcription of high affinity Mg^{2+} transport systems allowing the bacteria to overcome Mg^{2+} starvation.

The *M. tuberculosis* PhoP shows sequence similarity to the *S. typhimurium* PhoP response regulator[35]. In 2001, Perez *et al.* created an *M. tuberculosis* strain 103 mutant deleted in the PhoP–PhoR two-component system and showed that this mutant was unable to grow in murine bone marrow macrophages or in mice[36]. Complementation was performed using the response regulator and the wild type phenotype was restored when infected into macrophages, leading to the hypothesis that only *phoP* is

important for the virulence phenotype. This mutant is smaller in size and different than wild type in its cording properties when visually observed using the auramine (AFB) stain. In addition, mutant cells have an altered, rounded shape, and they show differences in levels of lipo-arabinomannan derivatives, compared to the wild type[37].

Another mutant in the *PhoP–PhoR* two-component system in *M. tuberculosis* H37Rv is also attenuated in macrophages and mice (S. Walters *et al.*, unpublished results). *In vitro* growth experiments using this mutant reveal an inability to grow in low Mg^{2+} containing medium, indicating that this system may be sensing Mg^{2+} and is the functional homologue of the *S. typhimurium* PhoP–PhoQ system in *M. tuberculosis*. Genes requiring PhoP for their expression during growth in broth have been identified by DNA array transcription analyses, and among these 85 are genes encoding proteins involved in lipid metabolism, cell wall synthesis, membrane transport, and oxidative stress response (S. Walters *et al.*, unpublished results). However, the binding of PhoP to these genes has not been demonstrated yet, and it is not known which, if any of them are direct targets for this response regulator. A similar number of genes show higher expression in the *phoP* mutant, and the observation that many of these are also induced in hypoxic conditions and other stresses suggests that the absence of PhoP is, in itself, a severe stress condition. Current experiments are attempting to find which genes controlled by PhoP are necessary for virulence.

MprA–MprB (Rv981–Rv982)

This two-component system was inactivated by Zahrt *et al.* and the mutant strain was tested for its ability to survive in activated and non-activated J774 macrophages and murine bone marrow-derived macrophages (BMM)[31]. The *mprAB* mutant grew better in non-activated macrophages than wild type, but displayed a similar survival phenotype to wild type in activated macrophages. In mice, the growth of the mutant is attenuated during the acute phase of infection in the spleen but not in the liver or in the lung. However, specifically in the lung and the spleen but not in the liver, the mutant failed to maintain viability and persist in the later stages of infection. These results collectively supported the role of the MprA–MprB two-component system in the establishment and maintenance of persistent infections in *M. tuberculosis*. The different phenotypes displayed by this mutant suggest that the histidine kinase MprB is sensing a tissue-specific ligand present during the many stages of the infection process or is able to respond to multiple signals. Future work on this interesting system will hopefully identify the stimulatory signal of MprB and the genes contained in the MprA regulon, which will provide many insights into the later stages of the *M. tuberculosis* pathogenic process.

PrrA–PrrB (Rv903c–Rv902c)

By screening a transposon mutant library of *M. tuberculosis* Erdman for the presence of two-component system mutants, Ewann *et al.* isolated a transposon insertion in the promoter region of the response regulator *prrA*[38]. Since the separation between *prrA* and *prrB* is 14 bp, disruption of the *prrA* promoter causes a polar mutation on *prrB*, essentially mutating the entire system.

The *prrA* mutant was used to infect murine bone marrow macrophages and BALB/c mice and displayed a defect in early (day 1–6) intracellular growth in macrophages. This phenotype did not affect the final course of the infection, since a full growth capacity was restored after one week, and at nine days post inoculation the mutant showed a bacterial burden inside the macrophages that was similar to that observed for the wildtype strain. Complementation of the macrophage phenotype was performed by the re-addition of the *prrA–prrB* system into the *prrA* mutant.

When infected into mice, the *prrA* mutant was not attenuated. Promoter analysis of *prrA* showed an induction in *M. bovis* infected macrophages for the first four days of the infection and a gradual decrease in promoter activity. These results are consistent with the findings of Graham and Clark-Curtiss who isolated *prrA* cDNA from intracellular *M. tuberculosis* but not from *M. tuberculosis* broth cultures, but inconsistent with the findings of Deretic *et al.* that did not observe induction of this system in macrophages[39,31].

RegX–SenX3 (Rv0491–Rv0490)

These genes were found to be polycistronic with the promoter region preceding[40] *senX3*. Biochemical experiments have shown that SenX3 can catalyse autophosphorylation, and that the phosphotransfer reaction occurs with[41] RegX3. Both unphosphorylated and phosphorylated RegX3 can bind to the DNA region directly upstream of *senX3* with over-expression of *senX3–regX3* in *M. smegmatis* increasing *senX3* expression. The potential RegX3 binding region in the *senX3* promoter is a palindromic sequence separated by one nucleotide consisting of GCTGTTTG located 101 bp from the translational start codon. These results suggest that RegX3 activates the expression of the *senX3-regX3* operon, and through this auto-regulation, signal amplification can occur. It is not currently known which environmental signal SenX3 is sensing or the genes that RegX3 controls, however, the role of this system in virulence has been tested. A transposon insertion in the *regX3* gene in *M. tuberculosis* Erdman has been created and the mutants ability to survive in bone marrow macrophages and mice proved to be similar to wild type[38]. Additionally, analysis of the *M. tuberculosis senX3* promoter expression during an infection of *M. bovis* BCG in macrophages showed no increase in expression during 14 days of infection.

TrcR–TrcS (Rv1033c–Rv1032c)

The histidine kinase TrcS can directly phosphorylate the response regulator TrcR, and similar to the *regX3–senX3* system, autoregulates its own gene expression though binding of the phosphorylated TrcR to the *trcR* promoter region[42,43]. Mutational analyses of this interaction and DNAse footprinting have identified an A-T rich sequence that is essential for TrcR binding and *trcR* regulation. The stimulatory signal for activation of the TcrR–TrcS system is currently unknown, but one study comparing the global transcription profile of an *M. tuberculosis trcS* mutant with *M. tuberculosis* H37Rv growing exponentially in 7H9 medium, found 36 genes expressed at a higher level in the wildtype, while 14 genes were overexpressed in the mutant[44].

Transcriptional disruption of the *tcrS* gene in *M. tuberculosis* H37Rv by homologous recombination and by transposon insertion in *M. tuberculosis* Erdman has been performed separately by two laboratories[38,45]. These *tcrS* mutants leave the upstream *trcR* gene intact, and presumably still functional, since in many examples, unphosphorylated response regulators are able to bind DNA and cause gene expression, albeit at lesser levels than when phosphorylated[46–52]. Therefore, these *trcS* mutants are not ideal to fully study the function of the *trcR–trcS* system in *M. tuberculosis* without the accompanying *trcR* mutant. These mutants may still retain many wildtype functions even though the systems ability to react to the initial stimulus is detached from the response in TrcS absence. Both mutants were tested for survival *in vivo*, and neither strain was attenuated in macrophages or in immunocompetent mice. Interesting, when the *tcrS* H37Rv mutant was infected into SCID mice, a hypervirulence phenotype occurred, with the mutant causing a significant increase in the time to death of infected mice[45]. The most direct and logical explanation for this phenomenon is that this two-component system's ordinarily function to repress genes necessary for bacterial pathogenicity, and this new class of mutant presents exciting possibilities concerning *M. tuberculosis* pathogenesis.

DosR–DosS (Rv3133c–Rv3132c)

DosR, also known as DevR, has been studied in *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* (reviewed by Park *et al.* and references, therein)[53]. It is required for the expression of a regulon containing approximately 50 genes that is induced during hypoxia or anoxia during hypoxic induced dormancy/persistence[53] and during NO stress (M. Voskuil, pers. commun.). Among the DosR requiring genes induced under these conditions are: *hxpX*, encoding the α-crystalline chaperone-like protein; *narX* and *narK2*, annotated as encoding respectively, a fused nitrate reductase and a nitrite extrusion protein; and *fdxA*, encoding ferredoxin, a protein involved in alternative respiratory pathways. DosR binds to a consensus sequence directly upstream of the promoter region of some of the genes, including *hspX* that require DosR for their induction under the conditions mentioned above[53]. There are two binding sites for DosR in the *hspX* promoter region, one at nucleotide positions – 110 to – 91 and the second, – 53 to – 34, relative to the transcription initiation nucleotide. Mutating the upstream site lowered *hxpX* expression after anoxic stress approximately 50% while mutating the downstream sequence essentially abolished all the induced *hspX* transcriptional activity. This data strongly suggests that DosR is an activator of *hspX*, presumably by interacting with the two DosR-binding sites, which in turn facilitates the binding of RNA polymerase to the *hspX* promoter. A *M. tuberculosis dosR* mutant showed an interesting virulence phenotype in that it initially grows better than the *M. tuberculosis* H37Rv parent strain in activated murine macrophages and immunocompetent mice. The mutant also kills SCID mice more rapidly than the wildtype[45], suggesting that the DosR–DosS system also functions to repress genes needed for pathogenesis as it is postulated for the TcrR–TcrS system.

KdpE–KdpD (Rv1028c–Rv1027c)

Genes annotated as being part of the *M. tuberculosis* K^+ uptake machinery are induced by low K^+ levels[54]. The two-component system KdpE (reponse regulator), KdpD (histidine kinase) is believed to control K^+ acquisition components in *M. tuberculosis* as related proteins do in other bacteria[55,56]. An analysis of the interactions between the sensing domain of KdpD with other unknown proteins was performed using yeast two-hybrid and three-hybrid systems and verified using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry[54]. These elegant studies shown that the sensing domain of KdpD interacts with two membrane lipoproteins, LprF and LprJ, and that a ternary complex can be formed between the histidine kinase domain of KdpD, and LprF, LprJ and the KdpD sensing domain. This interaction identifies the LprF and LprJ lipoproteins as the first accessory, adapter or ligand-binding proteins associated with a *M. tuberculosis* two-component system and leads to the speculation that these proteins may function to modulate the kinase or phosphatase activity of KdpD. Additionally, using yeast two hybrid assays these authors also found a direct interaction between KdpD and KdpE.

In the genome of *M. tuberculosis*, the *kdpD–kdpE* genes are adjacent to but divergent from the *kdpFABC*, a gene cluster believed to be involved in K^+ acquisition. In *E. coli*, the *kdpFABC* operon codes for a four-subunit, K^+-transporting P-type ATPase consisting of the KdpA, KdpB, KdpC and KdpF proteins, and this operon is induced during osmotic stress[56,57]. Overexpression of LprF and LprJ in *M. tuberculosis* increased expression of the low K^+ induced *kdpFABC* and mutating them modu-

lated expression of this regulon. While direct interactions of KdpE with the promoter region of these genes have not yet been demonstrated, these experiments indicate that KdpE is transcriptionally modulating their expression and may also auto-regulated the gene expression of *kdpD–kdpE*.

The KdpD–KdpE two-component system role in *M. tuberculosis* pathogenesis has been investigated, and a *M. tuberculosis kdpE* mutant shown normal growth in human macrophages (S. Walters *et al.*, unpublished results), and another *kdpE* mutant exhibited a hyper-virulent phenotype in SCID mice[45].

Other two component system regulators

Several other mycobacterial two-component system regulators have been mutated, but less is known about their function or the genes they control, compared to those already discussed above. Parish *et al.* created mutants in the *trcXY*, *narLS*, and the sole histidine kinase *Rv3220*, and tested the mutants' ability to grow in SCID mice. Both, *narLS* and the *Rv3220* mutants displayed a similar phenotype as wild type, while the *trcXY* mutant killed these mice quicker than wild type, adding it to the list of hyper-virulence mutants identified by this group.

Compared to other bacteria, *M. tuberculosis* contains relatively few two-component systems. For example, there are 62 two-component systems in *E. coli*[58], 27 in *Streptococcus pneumoniae*[59], 70 in *B. subtilis*[60], 74 in *Streptomyces coelicolor*[61], but only 11 paired systems in *M. tuberculosis*[30]. Since the primary residence of *M. tuberculosis* in the human lung, perhaps a less adaptive signaling repertoire may be required to survive compared to organisms like *S. coelicolor* that inhabit many different environmental niches. For *M. tuberculosis*, possibly only a few systems are required for a successful infection and through cross communication and unidentified accessory proteins, the different systems can coordinate their responses and further increase the complexity of the regulation of their signaling.

The next step in this exciting field of research is just beginning with the determination of the ligands stimulating the phosphotransfer reaction and the regulated genes of the *M. tuberculosis* two-component systems. Only recently, in a few examples, has there been identification of what the individual histidine kinases are sensing, and the genes the response regulators are controlling[44,53,54]. Identification of the activation signals of the two-component system involved in virulence, such as the PhoP–PhoR, MprA-MprB, and the PrrA–PrrB systems, will provide valuable information about the host environment, leading to a better understanding of the host–pathogen relationship. Additionally, discovery of the genes regulated by the response regulators of such systems are equally interesting, since they are the bacterial response to these signals. From examples in other bacterial systems, such as the *S. typhimurium* PhoP–PhoQ system, many of the genes regulated by response regulators involved in virulence are themselves virulence factors, acting in concert to create the proper bacterial response to the host. Therefore, with the identification of the *M. tuberculosis* systems involved in virulence and their target genes, many addition virulence factors in *M. tuberculosis* will be revealed paving the way for future exciting research.

Ser/Thr kinases

Many signals that indicate the inner status or the environmental surroundings of bacteria are integrated in the cell by a network system of phosphorylation and desphosphorylation of proteins. Besides the 13 histidine kinases that are part of the two component systems, the *M. tuberculosis* genome is annotated to contain 11 eukaryotic-type serine/threonine kinases (named PknA, B, D, E, F, G, H, I, J, K and L), 36 phosphatases, eight phosphotransferases and at least one kinase inhibitor that are possibly components of the bacterial signaling network. Analysis of the eukaryotic like kinases in other microorganisms indicated that these kinases are components of signaling pathways involved in secondary metabolism and morphogenesis[62,63]. Moreover, it has been shown that they are also important virulence determinants and fundamental components of the bacterial stress response[64]. In *M. tuberculosis*, the ser/thr kinases are a largely unexplored family. By biochemical and structural analysis it was determined the location and the activity of several of these kinases, but there is not much information about possible substrates or pathways.

Expression of *pknA* in *E. coli* induced the elongation of cells indicating that probably this kinase is possibly involved in the control of morphological changes of the bacterium[65]. The crystal structure of PknB has been resolved and it has been determined that it is a transmembrane kinase. Since *pknB* is located in the same operon as *pknA*, it has been suggested that this kinase is also involved in the process of cell elongation. However, the over-expression of *pknA* alone in *E. coli* is sufficient to induce cell elongation in this microorganism. A gene encoding a ser/thr phosphatase is also part of the same operon with *pknA* and *pknB*. The kinases PknF and PknG have been also characterized[66]. PknF is a trans-membrane protein and it has been speculated that the carboxyl terminal region of this kinase might be involved in environment sensing[66]. PknG is cytosolic and has a structure similar to the *Yersinia* YopO. The *Yersinia* YopO is secreted, and it can mediate morphological alterations of infected cultured HeLa cells[67]. However, the effect, if any, of *M. tuberculosis* PknG on the signaling machinery of eukaryotic cells has not been determined.

For the rest of the ser/thr kinases, no evidence exists concerning their possible functions and only there are speculations according to the location of each gene in the

genome of *M. tuberculosis*[68]. *pknD* resides near other genes that code for the components of a phosphate-uptake system. The gene that encodes PknK is located near a putative transcriptional regulator and the carboxy-terminal region of PknK is itself homologous to some members of the LuxR family of transcriptional regulators[68]. It was suggested that PknG and PknH are involved in the process of glutamine uptake induced under nitrogen. *pknI* is in the same operon that the genes that encode for a probable D-amino acid hydrolase (*Rv2913c*) and a signal recognition particle (*ffh*). It has been suggested that these proteins are involved in cell division[68]. *pknL* is in the same operon as a putative transcriptional regulator. *pknF*, *pknE* and *pknH* are in or near operons that code for ABC transporters. *embR* is part of the same operon with *pknH*. EmbR is a transcriptional regulator that regulates *embB* and *embA*, two cell wall arabinosyltransferases that are target of the drug ethambutal[69]. Future studies of this family of eukariotic kinases should elucidate their role in the signaling pathways that couple external and internal signals with the transcriptional machinery of *M. tuberculosis*.

Sigma factors

Among the most important bacterial transcription activators are the sigma factors. By binding to the RNA polymerase, sigma factors give to the core enzyme, containing the catalytic subunits the specificity for a particular promoter. This specific interaction between enzyme and promoter region provides a means of regulating gene expression in response to various environmental conditions as new sigma factors bound to RNA polymerase allow different groups of genes to be expressed[70].

Analysis of the *M. tuberculosis* genome sequence indicates that this pathogen has 13 sigma factors[30] that are members of the Sigma70 family because of the similarity that they share with the Sig70 from *E. coli*. According to the phylogenetic relationship the Sig70 family is divided into 4 or 5 groups[71]. Group 1 includes principal and essential sigma factors. In *M. tuberculosis* the group 1 is represented by SigA that most probably controls housekeeping genes[72,73]. SigB is the representative member of group 2 that contains sigma factors closely related to the primary sigma factor but are non-essential[70]. Expression of *sigB* in *M. tuberculosis* can be up-regulated under some stress conditions (see below). Group 3 comprises alternative sigma factors that are more distantly related and respond to specific signals. In *M. tuberculosis*, SigF represents this group and shows similarity to the alternative sigma factors SigF of *S. coelicolor* and sigB and SigF of *B. subtilis*. In these bacteria, SigF is involved in sporulation[74,75] and in *B. subtilis*, SigB controls responses to general stress[76]. The other 10 sigma factors are highly diverged from the Sigma70 and belong to the group number 4. These sigma factors are designated as the extracytoplasmic function (ECF) family that is involved in responding to external conditions[77].

Little information is available concerning the functions and regulation for most *M. tuberculosis* sigma factors. Manganelli *et al.* using RT–PCR with molecular beacons have studied the expression of 10 different sigma factors (*sigA*, *sigB*, *sigC*, *sigD*,*sigE*, *sigF*, *sigG*, *sigH*, *sigI*, *sigM*) during exponential phase and under several stress conditions[78]. In this work it was shown that the genes for all sigma factors were expressed during the exponential phase of bacterial growth, with *sigC* showing the highest levels of expression. When the bacteria were treated with non-toxic levels of sodium dodecyl sulphate (SDS) *sigC* and *sigM* were down-regulated and *sigB* and *sigE* up-regulated. Heat shock treatment down-regulated *sigC* and *sigG* but up-regulated *sigB*, *sigE* and *sigH* transcription. Mild cold shock or room temperature induced the expression of *sigI* and down-regulated *sigG*. Incubation of the bacteria with water did not affect *sigI* expression but decreased mRNA levels of the rest. Low aeration conditions (exponential growth cultures incubated 24 h at 37°C without agitation) or stationary phase (bacteria collected after 70 h at OD > 2) decreased mRNA levels of almost all sigma factors. In stationary phase only *sigB*, *sigE* and *sigF* remained constant and under low aeration, *sigE* and *sigF* remained constant but the transcription of *sigB* was significantly up-regulated. Hu *et al.* demonstrated that the expression of *sigI* and *sigJ* was prominently up-regulated in stationary phase when the bacteria were maintained for 100 days in standing cultures. In these conditions levels of *sigG*, *sigH*, *sigD*, *sigF*, *sigL* and *sigK* did not change but *sigA*, *sigB*, *sigC*, *sigE* and *sigM* were down-regulated[79]. These results may suggest a predominant role of SigI and SigJ in stationary growth phase conditions. Graham *et al.* had compared the expression *in vitro* and *ex-vivo* of the different sigma factors of *M. tuberculosis* using the selective capture of transcribed sequences method (SCOTS). These workers found that *sigA*, *sigB*, *sigD*, *sigE*, *sigF*, *sigG*, and *sigH* but not *sigC* where expressed when bacteria were growth in broth culture. When *M. tuberculosis* were growing inside macrophages, the expression of *sigA*, *sigB*, *sigE*, *sigF* and *sigH* [39] could be detected.

SigA

The *sigA* genes of *M. smegmatis* and *M. tuberculosis* were thought to encode the principal sigma factors in these organisms by their primary amino acid sequence similarity to SigA, the principal factor of *Streptomyces* spp as well as their similarity to each other[80,81]. Moreover, *sigA* was shown to be essential in *M. smegmatis*[82] and in *M. tuberculosis* (J. Timm and I. Smith, unpublished results). As expected the promoter sequences recognized by RNA polymerase containing SigA are similar to those recognized by *E. coli* and *B. subtilis* RNA polymerases containing the principal sigma factor in these organisms[81].

SigA is also involved in virulence as *M. bovis* strain TMC403 that is attenuated in a guinea pig model contains a missense mutation (R515H) in the *sigA* (*rpoV*) gene[83]. This mutation is adjacent to region 4.2 that interacts with the promoter consensus-35 region. Mutations affecting transcription of specific genes have been described in the same region adjacent to region 4.2 in the major sigma factors of *E. coli* and *B. subtilis*[84,85]. These mutations, which have no effect on *in vitro* growth are postulated to prevent interaction between the major sigma factor and several positive activators. The phenotype of the *M. bovis sigA* R515H mutation suggests that there is an activator that interacts with SigA to transcribe *M. bovis* genes that are essential for pathogenicity. WhiB3, discussed below, seems to play this role[86].

SigF

SigF, is an alternative sigma factor member of the group 3, and according to experiments performed in *M. bovis* BCG, the expression of this sigma factor is highly induced in stationary phase (when bacteria were grown with shaking until OD > 2) and also under nitrogen depletion[87]. A null mutant of *sigF* in *M. tuberculosis* was not attenuated in any of these conditions or when infecting macrophages. However, the *sigF* mutant was more sensitive to the treatment with the antibiotics rifampin and rifapentine and it was attenuated in an experimental model of infection in mice[88]. Genes under SigF regulation have been determined by DNA microarray (W. Bishai, pers. commun.). Previously, it has been reported that the expression of the 16-kilodalton alpha-crystallin protein (acr) in early stationary phase is under SigF regulation[89]. In the reports discussed above there is some discrepancy about the conditions under which some sigma factors are transcribed, particularly, with reference to the expression of *sigF* and *sigB* in stationary phase and low aeration. It is difficult to compare the different studies since definition of these conditions varies from one work to the other. Another complication is that the importance of changes in transcriptional levels of the sigma factors may be relative since other modes of regulation may also be relevant, i.e. post translational control, as discussed below.

SigE, SigH and SigB

Recently, the response of SigE, SigH and SigB to different stresses has been extensively studied[90–92]. The availability of mutant strains and the use of DNA microarray technique have revealed that there is a transcriptional regulation circuit generated by these sigma factors. As it was described above, the expression of SigE is up-regulated under heat shock, cell wall stress and oxidative stress. A *sigE* mutant is more sensitive to SDS, oxidative stress and heat shock[78]. Using molecular beacons and DNA microarrays, Manganelli *et al.* have defined the SigE regulon under exponential growth and cell wall stress[90]. These studies demonstrated that under non-stress conditions the expression of 38 genes is dependent on SigE. Interestingly, only one gene, *sigB* that required SigE for its expression in exponential growth, had an EFC-sigma-like promoter sequence. The results of this array indicate that during exponential growth SigE, there are indirectly regulated housekeeping genes. After SDS treatment, 62 genes were induced in the wild type strain. The high expression of 23 genes in 13 transcriptional units requires SigE. A sequence that resembles an ECF sigma promotor preceded nine of these sequences. SigE differentially regulates genes involved in mycolic acid synthesis and fatty acid metabolism, as well as transcriptional regulators and there are a many other genes annotated as unknown. The role of these genes in the virulence of the bacteria remains to be elucidated by disrupting them and evaluating the virulence of the mutant strain.

sigB is also under SigE regulation during cell wall stress[90]. Recently, using DNA microarrays we compared gene expression of a *sigB* mutant with the wild type strain during SDS treatment. We demonstrated that only two genes, out of the 20 that are under SigE control after SDS treatment also require SigB for their maximum induction (P. Fontan *et al.*, unpublished results). These two genes are annotated to code for a small heat shock protein and a possible transcriptional regulator. The relevance of these genes for the virulence of the *M. tuberculosis* remains to be determined. SigH is the third member of the circuit. This sigma factor is the ortholog of SigR from *S. coelicolor*[93] and controls the expression of genes needed for bacteria to tolerate heat shock or oxidative stress[94]. A *sigH* mutant of *M. tuberculosis* was sensitive to heat shock, and to different oxidative stresses like hydrogen peroxide, organic peroxide and diamide (thiol oxidant)[91,92]. Microarray analysis indicates that SigH is not relevant for expression of genes during bacterial exponential growth, but SigH controls the transcription of 39 genes when bacteria are suffering redox stress induced by diamide, including *sigB* and *sigH*[91]. The induction of thioredoxin and thioredoxin reductase in a SigH dependant manner suggests that this is the system that mycobacteria used to maintain the redox homeostasis. The induction of *sigE* expression by diamide and the control of this expression by SigH have been described in two different reports. However, no *sigH* consensus promotor has been found upstream of *sigE* (Fontan *et al.*, unpublished results).

Interestingly analysis of the promotor region of genes under the control of SigE and SigH suggests that both sigma factors recognize very similar consensus sequence[91]. Thus, the same promoter is used by SigE under stress and non-stress conditions and by SigE under stress to induce the transcription of *sigB*. It is intriguing how promoter discrimination occurs between these two sigma factors. It can be speculated that a subtle changes in the sequence

are responsible for initiation of the transcription using one or another sigma factor. Another possibility is that each sigma factor is differentially available depending on the interaction with their specific antagonists, the anti-sigma factors.

The expression of sigma factors can be regulated at the level of synthesis, proteolysis or by the interaction with their anti-sigma factors[77]. In *M. tuberculosis* only the regulation of SigF has been carefully analysed[95]. Beaucher *et al.* have shown that SigF activity is regulated by reversible interaction with UsfX. This anti-sigma factor is the product of the *usfX* gene positioned directly upstream of the *sigF* gene. These authors also identified two antagonists of UsfX, the anti–anti sigma factors and demonstrated that one of these, RsfA is regulated by redox potential and they suggest that the second, RsfB may be regulated by phosphorylation. The postulated model for SigF and its regulators indicates that SigF remains bound to UsfX until bacteria are subject to reducing conditions, like the possible environment inside the granuloma. When the redox conditions changes, RsfA becomes active and binds UsfX liberating SigF. The role of RsfB is not clear and the kinase involved in the putative phosphorylation of this anti–anti-sigma factor has not been described yet. Three other proteins of *M. tuberculosis* are similar at the level of primary sequence to RsrA, the anti-sigma factor of SigR in *S. coelicolor*. The genes coding for these proteins are downstream of *sigH*, *sigE* and *sigL*. In each case the protein encoded by the putative anti-sigma factor specifically binds to and reversible inactivates the cognate sigma factor (S. Rodrigue *et al.*, unpublished results). Two more genes, *Rv1904* and *Rv2638* have been annotated as similar to an anti–anti-sigma factor of SigB in *S. coelicolor*, but the correspondent anti-sigma and sigma factors have not been identified yet.

The contribution of these global transcriptional regulators to the bacterial pathogenicity has been demonstrated *in vitro* and *in vivo*. The *sigE* mutant was impaired for growth in macrophages[90] and in mice (R. Manganelli *et al.*, unpublished results). *M. tuberculosis* strains carrying mutated *sigF* or *sigH* genes did not show any attenuation when used to infect macrophages but were demonstrated to be attenuated in different models of mice infection[88,96]. Animals infected with either *sigF* or *sigH* mutants did not show a reduced number of bacterial colony forming units (cfu) in the lung when compared with the cfus in the lungs of animal infected with the wild type strain. However, animals infected with a *sigH* mutant showed reduced histopathological signs of lung damage and the time-to death analysis of mice infected with *sigH* or *sigF* mutants was greater when compared with animals infected with the parental strain. These observations suggest that some genes of the regulons under the control of these sigma factors are important bacterial virulence determinants although the survival of the pathogen inside the host is not affected by the absence of these proteins.

Accessory transcriptional factors

WhiB family

WhiB is a transcriptional regulator that has been shown to be essential in *S. coelicolor* for the sporulation of aerial hyphae. Interestingly, this family of transcriptional regulators is present in all actinomycetes but not in other organisms[97]. WhiB has seven homologs in *M. tuberculosis*, and this family is present also in *M. leprae* and *M. smegmatis*. The function of these regulators in mycobacteria is intriguing since these microorganisms do not sporulate. One speculation is that in mycobacteria the *WhiB* genes can encode proteins related to the dormancy state of the bacteria. However, a *whiB3* null mutant in *M. smegmatis* did not show any defect when it was evaluated in the Wayne model for dormancy state[98]. It was demonstrated in *M. smegmatis* that *whiB2* is an essential gene[82]. By constructing an *S. smegmatis* strain with a disruption in the *whiB2* gene but with an extra-chromosomal copy of this gene under an inducible promotor, Gomez *et al.* demonstrated that WhiB2 has a role in cell division and septation[82]. In *M. tuberculosis*, the expression of this gene was observed to be up-regulated when bacteria infected macrophages. As it was described above, a point mutation, a change in Arg-515 to His, in the 4.2 region of SigA was shown to be responsible for the loss of virulence of *M. bovis*[83]. Steyn *et al.* have demonstrated that in *M. tuberculosis* WhiB3 interacts with the 4.2 region of SigA and that the lost of virulence of the *M. bovis* mutant strain is due to the lack of interaction of WhiB3 with SigA[86]. Strains with a mutated *whiB3* gene have been constructed in *M. bovis* and *M. tuberculosis*[86]. The mutant strain in *M. bovis* was attenuated in a guinea pig infection model. Evaluation of the *M. tuberculosis* mutant strain in different animal models of infection did not show any difference in bacterial load in different organs. Interestingly, mice infected with the mutant strain showed less tissue damage specifically in the lungs and the survival of the animal was significantly increased when compared with animals infected with the wild type strain.

Transcriptional repressors

In the *M. tuberculosis* genome there are 31 proteins annotated as transcriptional repressors. For many of these proteins the only information available is the homology at the sequence level with transcriptional repressors characterized in other microorganisms. However some of these regulators have been well characterized by several laboratories and their roles in the regulation of the global

gene expression has been studied during different environmental stresses.

IdeR

IdeR is the main regulator of iron metabolism in *M. tuberculosis*[99]. This transcriptional regulator was identified first as a repressor but recent work from our laboratory has demonstrated that IdeR is also an activator. Under high intracellular iron condition, IdeR remains bound to the iron box present in the promoter region of genes under its regulation. Thus, under high intracellular levels of iron, IdeR simultaneously represses the *mbt* operon involved in the synthesis of the siderophore mycobactin and activates the genes *bfrA* and *bfrB* that encode iron storage proteins[100,101]. The functional mechanisms of IdeR, as well as the complete regulon under IdeR control have been reviewed recently by Rodriguez and Smith[102].

LexA and RecA

LexA and RecA are two regulatory proteins involved in the bacterial response to DNA damage[103]. When the DNA damage occurs, RecA binds to the single stranded DNA region and induces the autocatalytic cleavage of LexA, then LexA can no longer remain bound to the so-called SOS boxes found upstream genes of the SOS regulon. In a recent work, Davis *et al.* have defined the LexA binding motif in *M. tuberculosis*[104]. In the same work by using microarray analysis these authors demonstrated that the regulon under LexA control comprises 15 genes. Ten of these genes were not known to be induced by DNA damage. Interestingly, some genes known to be under LexA control in *E. coli* were not induced with mitomycin C (an inducer of DNA damage) in *M. tuberculosis* and some others induced genes did not show a LexA-binding motif in their promoters. This last observation indicated that LexA activation is not the only regulatory mechanism of the SOS response in this pathogen.

HspR and HrcA

In *M. tuberculosis*, proteins involved in the heat shock response are negatively controlled by two transcriptional repressors, HspR and HrcA[105]. HspR is homologous to the repressor that controls the Hsp70 operon in *Streptomyces*[106]. HspR in *M. tuberculosis* controls the expression of the Hsp70 operon and in some extent the expression of GroES[105]. A mutant strain of *M. tuberculosis* with a deletion of *hspR* was attenuated when it was used to infect mice[107]. The authors suggest that this phenotype is probably due to an enhanced response of the host's immune response caused by over expression of the Hsp70. HrcA in *B. subtilis* controls both *hsp60* (*groEL*) and *hsp70* heat shock systems[108]. The homologous protein in *M. tuberculosis* mainly regulates the expression of the *hsp60* family of heat shock proteins (*groEL1*, *groEL2* and *groES*). Interestingly, the heat shock response in *M. tuberculosis* is under both, positive and negative regulation. At least three sigma factors, SigH, SigE and SigB are involved in the positive control of this response. SigH seams to be the main regulator controlling the expression of the *hsp70* operon and *clpB*[92]. SigH also controls *sigE* and *sigB* expression under heat shock stress conditions[78]. Preliminary results from our laboratory indicate that under heat shock stress SigB partially controls the expression of the *hsp70* operon, *acr2* and the *groEL/groES* chaperons (P. Fontan *et al.*, unpublished results). Since, SigE controls *acr2* expression when bacteria is treated with diamide, it will be interesting to determine if the sigE also controls the expression of this chaperon or others under heat shock stress.

RelA

As discussed elsewhere in the introduction, *M. tuberculosis* is able to persist in a slow growing or non-growing 'latent' state for long periods in infected hosts. The bacterium must be able to shut down or down regulate the synthesis of those factors that are required for exponential growth, e.g. ribosomes, tRNAs, etc. At the same time, new components must be synthesized to allow bacterial survival under these conditions, e.g. there are marked changes in the cell wall when *M. tuberculosis* is maintained for long period under limited oxygen conditions[109], etc. The primary *in vitro* model used to study the phenomenon of latency has been the Wayne model in which bacteria are exposed to limited oxygen levels[110]. DosR (DevR), the two-component response regulator discussed previously, is induced under these conditions and is required for the up-regulation of other genes induced during anoxia[53,111]. Global *M. tuberculosis* gene expression with DNA arrays has been analysed after exposure to partial or complete nutrient starvation[112], as it is thought that these conditions may mimic the environment to which the bacterium is exposed at later stages of infection. In the course of this work, it was found that genes encoding ribosomal proteins, enzymes involved in intermediary metabolism, energy production and lipid biosynthesis, as well as other genes were all repressed. Significantly, *relA*, encoding (p)ppGpp synthase and some other regulatory proteins were induced by nutrient starvation. The RelA result was not unexpected since much work in other prokaryotes has shown that the regulatory nucleotide (p)ppGpp, the product of the enzymatic function, of this protein is to down-regulate the expression of genes for stable RNAs, ribosomal proteins and lipid biosynthetic enzymes among others, during nutrient starvation, while it activates other genes[113]. The mechanism by which (p)ppGpp selectively inhibits gene expression, while it activates others is unknown, but there is

some evidence that it binds to the β–subunit of RNA polymerase (reviewed in ref. 114).

The importance of (p)ppGpp for the late growth regulation in mycobacterial species had previously been observed, using conditions of carbon limitation and overproduction of the regulatory nucleotide[115]. Recent experiments with a *relA* mutant of *M. tuberculosis*[116] have confirmed the importance of (p)ppGpp in allowing *M. tuberculosis* to survive during stationary growth caused by nutrient limitation or during mouse infections[117]. The mutant shows decreased survival during stationary growth and in mouse lungs and spleens. DNA array analyses also indicated that during the normal limitation of growth caused by nutrient limitation, RelA and presumably its enzymatic product are responsible for down regulating genes for the translational apparatus. At the same time several other genes were higher in the *relA* mutant compared to the wild type strain during nutrient starvation[117]. These exciting results provide many new potential targets for possible antitubercular therapies.

Conclusions

The relatively recent availability of tools to analyse *M. tuberculosis* at the molecular level had made possible a great advance in the understanding how this microorganism uses its cellular signaling and transcriptional machinery to survive different stresses. As we describe here, there is growing evidence for complex connections between the factors from the different regulatory circuits. These connections are certainly relevant under multiple simultaneous stress conditions, which is more related to the *in vivo* situation than to the carefully controlled single-stress situations usually studied in the laboratory. Now, taking advantage of the existing *in vitro* data and the available technology like DNA microarrays it will be possible to study the orchestrated response of *M. tuberculosis* during host infection. Hopefully, the identification of the regulatory components that contribute to the success of *M. tuberculosis* as a human pathogen will lead to the development of new drugs and vaccines to combat and prevent tuberculosis.

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