

Virulence and pathogenicity

The key role of *phoP* orchestrating *M. tuberculosis* virulence network

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The development of mycobacterial genetic tools makes possible today the inactivation of selected genes, allowing the study of the molecular basis of pathogenicity in *Mycobacterium tuberculosis*. In addition genomic studies permit the comparison of virulent and avirulent *M. tuberculosis* strains.

We were interested in the study of the two component system *phoP/R* of *M. tuberculosis* complex due to the increased expression of transcription factor gene *phoP* in a particular strain which caused untreatable XDR TB outbreaks. In 2008 the sequence of strain *M. tuberculosis* avirulent H37Ra (derived from the virulent H37Rv) has been annotated. H37Ra contains a single point mutation in the amino acid in PhoP 219 changing a serine by leucine, conferring the loss of lipid complex and export of ESAT 6.

The construction of an *M. tuberculosis phoP* mutant by homologous recombination demonstrated the essential role of *phoP* in *M. tuberculosis* virulence. The *phoP* mutant showed a phenotype with drastic alteration of the morphology of bacillus and loss factor coding associated with the virulence of the bacillus. In addition the gene inactivation *phoP* shows extreme attenuation in macrophage and in different mouse models used.

PhoP regulates major changes in the morphology of *M. tuberculosis*, the secretion of the major T-cell antigen, ESAT-6, and the synthesis of pathogenic lipids. Altogether, these findings indicate that PhoP orchestrates a variety of functions implicated in the intracellular style of live *M. tuberculosis*.

The role of *Mycobacterium tuberculosis sigE* in physiology and virulence

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Mycobacterium tuberculosis (Mtb) survives in macrophages and usually subverts the bactericidal mechanisms of these phagocytes. The understanding of this host-pathogen interaction is relevant for the development of new treatments for tuberculosis. The adaptation of Mtb to the intracellular milieu depends on its ability to regulate the expression of its genes. Sigma factors are important bacterial transcription activators that bind to the RNA polymerase and give it promoter specificity

Mtb has 13 sigma factors, of which 10 are of the ECF sigma factor class that are important for control-ling genes that are involved in responding to environmental stresses. The role of many of these sigma factors in Mtb virulence has been analyzed, as mu-tants have been created with disruptions in Mtb sigma factor genes. However, only the sigE mutant is attenuated in resting and activated macrophages, suggesting that the product of this gene, sigma factor E (SigE), a member of the ECF sigma group, controls genes directly related to the intracellular survival of the bacteria. DNA array technology has allowed us to identify the SigE regulon during infection of human and murine macrophages and also to study the impact of this regulon on the transcriptional response of these host cells. Our results indicate that SigE regulates the expression of genes involved in the maintenance of Mtb cell envelope integrity and function during macrophage infection. Analysis of the macrophage transcriptome during this time indicates that the SigE regulon is involved in the modulation of the inflammatory response.

“Do we know which are the factors involved in the mycobacterial dormancy?”

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Latent tuberculosis indicates previous exposure to the tubercle bacilli by a positive skin test response together with the absence of signs of disease. During latency, the tubercle bacilli are considered to be dormant.

Dormancy has been identified as a *reversible state of low metabolic activity, in which the bacterial cells can persist for extended periods without division*. Therefore, dormant tubercle bacilli are considered to be in a non-replicating stage, establishing a close control on their cell division. In such a conditions, bacilli are refractory to detection and analysis.

Within the framework of EU-funded projects, we have studied several aspects of *M. tuberculosis* and *M. bovis* BCG thought to be related to persistence and dormancy. Some results are following:

- The *dosR/S* two-component system is considered controlling the dormant stage of the bacilli. The transcription of this system displays a complex regulation (Rodriguez, 2008).

- The chromosomal origin of replication of the *M. tuberculosis* genome is a hot spot for the insertion of IS6110 (Turcios, 2008). This IS could putatively be participating in the control of the bacterial cell replication.

- We search for the potential involvement of IS 6110 in the *M. tuberculosis* dormancy. At the moment, not apparent relationship was found (Otal, 2008).

- Mycobacterial lipids are more and more often involved in the persistence of bacilli. Current research is being focussed in this field.

Unravel the physiology of dormant bacilli are worthy required to help in the control of Tuberculosis.

Studies on iron acquisition and regulation in *Mycobacterium tuberculosis*

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Like most living organisms *M. tuberculosis* (Mtb) requires iron as a cofactor for enzymes involved in vital cellular functions. Due to the poor aqueous solubility of ferric ion (Fe³⁺) in the presence of oxygen and at neutral pH, free iron (III) is not found in the mammalian host, but is sequestered in complexes with iron binding proteins. Therefore, in order to establish a productive infection Mtb must be able to overcome the iron deficiency imposed by the host. When faced with iron limitation, Mtb produces high affinity iron chelators or siderophores named exomycobactins that sequester iron from the environment and are transported into the cell via specialized proteins. IrtA and IrtB, two membrane proteins belonging to the family of ABC transporters, are required for iron uptake and for normal replication of Mtb in macrophages and in mice. The process of iron acquisition and storage is tightly controlled to prevent surplus free iron that can catalyze the generation of toxic oxygen radicals. This role is performed in Mtb by the Iron Dependent Regulator (IdeR), an essential transcriptional repressor of iron uptake and activator of iron storage.

Since both iron acquisition and regulation are critical for Mtb infection a clear understanding of the molecular mechanisms involved in these processes may open new possibilities to combat this pathogen.

Fulfilling molecular koch's postulate for *Mycobacterium tuberculosis*: a new era of TB research

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Jean Antoine Villemin, a medic in Napoleon's army had observed that new recruits often developed consumption. He hypothesized that consumption (alias for TB) was an infectious disease and published a paper in 1868 demonstrating that inoculation of lung material from a cadaver that had died of consumption could cause a consumptive-like disease in rabbits. He postulated that consumption was an infectious disease. In 1871, Gerard Henrik Armauer Hansen visualized bacilli (*Mycobacterium leprae*) in a leprosy patient and postulated that these bacilli could cause leprosy. Although this was the first association of bacterium with a human disease, it was not till 1882 when Koch proved that bacilli could cause disease. By observing, purifying, and transferring the bacilli, Koch unequivocally established that the tubercle bacilli (alias *Mycobacterium tuberculosis*) caused tuberculosis. This knowledge allowed investigators to focus on bacilli for preventing and controlling tuberculosis. In 1944, Avery, MacLeod, and McCarty fulfilled Molecular Koch's postulate for the first time establishing that a phenotype (namely virulence) could be caused by a specific genotype encoded by DNA by 1) characterizing an

avirulent mutant, 2) cloning the virulence genes, and 3) transforming the avirulent strain to a virulent strain by transferring the virulence genes. In addition to transformation, the processes of conjugation and transduction allowed for the fulfillment of Molecular Koch's postulate in diverse bacteria but *Mycobacterium tuberculosis* remained genetically intractable. Using recombinant DNA technologies and mycobacteriophages, we were able to introduce foreign DNA into tubercle bacilli for the first time in 1987 using a shuttle phasmid. The subsequent isolation of mc²155, a plasmid transformable *M. smegmatis* mutant, allowed us to discover the previously unknown targets of Isoniazid and Ethionamide. Moreover, the further development of genetic tools for mycobacteria and the unveiling of the genome sequence of *M. tuberculosis* have allowed for elucidation of novel virulence mechanisms of tubercle bacilli. Despite the Darwinian consequence of strains of *M. tuberculosis* resistant to all TB drugs, the power of genetics (i.e. the Fulfilling of Molecular Koch's Postulate) in mycobacteria promise to lead to novel chemotherapies and immunotherapies to treat TB.

Mycobacterial and host factors in Tuberculosis

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To understand host-pathogen interactions in tuberculosis, we are developing approaches combining cell biology and functional genomics to understand the mechanisms of macrophage parasitism by *M. tuberculosis*. We used signature tag transposon mutagenesis (STM) and a strategy of allelic replacement to identify *M. tuberculosis* genes involved in the ability of the bacillus to parasitize human macrophages. The inactivation of the two two-component signal transduction system PhoP-PhoR has led to an attenuated phenotype. This system regulates positively a large number of genes including the synthesis of diacyl-trehalose (DAT), polyacyl-trehalose (PAT) and sulfolipids (SL). The role of these different lipids in virulence mechanisms still remain to be understood. However, several applications can already be considered from the identification of these virulence loci such as drug targets and vaccine candidates.

We are also studying early interactions between Mycobacteria and human phagocytes. The lectin DC-SIGN is the major *M. tuberculosis* receptor on human dendritic cells. The mycobacterial lipoarabinomannan (LAM) is a key DC-SIGN ligand. We have observed DC-SIGN induction in alveolar macrophages from patients with tuberculosis (TB). The functional consequences of DC-SIGN ligation by the bacillus in the lungs of infected individuals are being investigated. Moreover, a case-control study has shown that in a South-African cohort, a variant of the promoter of the gene encoding DC-SIGN is strongly associated with protection against TB. This result further highlights the important role played by the receptor during TB infection. The implication of other host factors are identified through transcription profiling analysis.

Virulence properties of *Mycobacterium bovis*

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M. bovis is the causative agent of bovine tuberculosis and it can infect numerous mammalian species including man. This bacteria belongs to the *Mycobacterium tuberculosis* complex and is highly related to *M. tuberculosis*, having both bacteria a sequence similarity of 99,95 % and a totally conserved sinteny. However the host range, and some biochemical properties, and cultural characteristics are different. The molecular basis for these differences are ascribed to genomics deletions (RD, regions of differences) in *M. bovis* respect to *M. tuberculosis* and to some SNPs. In addition, we have observed novel gene deletions and duplications in *M. bovis* genome.

The virulence of some selected *M. bovis* strains was evaluated in a mice model of tuberculosis. The strains were typed using spoligotyping and MIRU typing. A striking variability in lethality, infection, histopathology and immune response was observed. The most pathogenic strains come from wildlife. Using microarray technology we evaluated the transcriptome of the most and least pathogenic strains and important differences in gene expression were observed. Currently, we are evaluating the virulence of *M. bovis* in cattle by scoring the advance of TB in animals at slaughterhouses, and performing an age-stratified correlation with particular genotypes.