

News from the antituberculosis front at two recent European meetings

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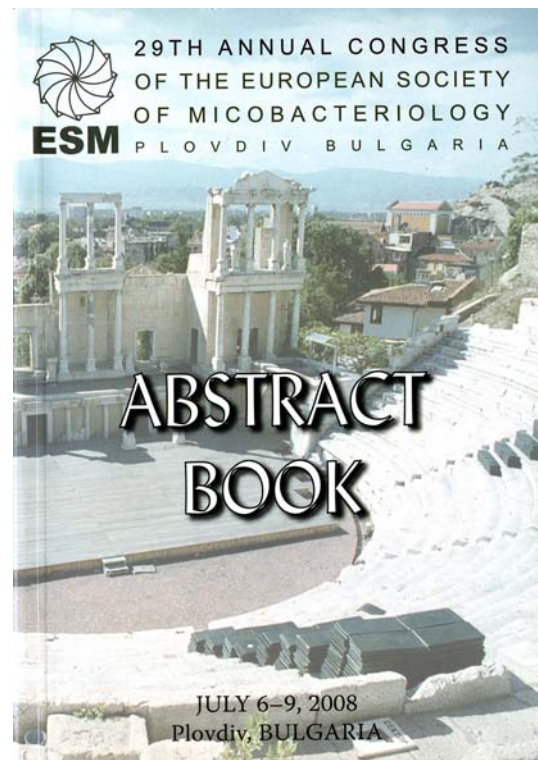
Introduction

The yearly conference of the European Society of Mycobacteriology (29th Annual Congress of the European Society of Mycobacteriology—July 6–9 2008—Plovdiv, Bulgaria) attracted more than 200 participants who exchanged recent knowledge on clinical aspects of mycobacterial biology and tuberculosis infection.

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The Saltsjöbaden-Conference on pathogenesis of mycobacterial infections (7th International Conference on the Pathogenesis of Mycobacterial Infections—June 26–29

2008—Saltsjöbaden, Stockholm, Sweden) is held every 3 years, its 2008 edition gathered some 200 participants who discussed molecular biology of mycobacterial research.

show various spread patterns dependent on geographical location, social environment and age group.

MDR-TB cases have been reported in all European countries: in 2005 the study of an MDR outbreak detected



Seventh International Conference on the Pathogenesis of Mycobacterial Infections

26-29 June, 2008 at Grand Hotel Saltsjöbaden, Stockholm, Sweden



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Here we report the highlights of both conferences (Plovdiv, abbreviated in the text as P; Saltsjöbaden, abbreviated in the text as S) combining their complementary approaches, together they offer an integrated view of the recent advances in the understanding of tuberculosis and on the possibilities to combat it.

Genetic and genomic methods in epidemiology and diagnosis of TB

The patterns of tuberculosis and antibiotic resistance spread

Tuberculosis, the disease caused by infection with *Mycobacterium tuberculosis* (TB), is no longer a problem confined to deprived areas or remote regions, the ease of travel and the mobility of the populations have modified the pattern of tuberculosis spread. The incidence of tuberculosis in a given geographical area results from the rates present in the native population and those contributed by immigrants. In low incidence countries as Sweden, in which the transmission of tuberculosis among the native population is low, the influx of infected immigrants has contributed to maintain the TB epidemic (E. Svensson, Gothenburg, Sweden. P). New isolates resistant to the commonly used antibiotics, including the difficult to treat MDR and the almost untreatable XDR strains (Box 1), are a serious threat for human health, not the least because they

in Austria, a country with low incidence of TB, serves to exemplify the importance of human migration and transmission patterns and to stress the need for adequate treatment and follow-up of the patients. In this case the outbreak was initiated by a patient who, due to his refugee status in the country, proved difficult to be followed. First diagnosed as suffering non-MDR pulmonary disease, the discontinuity in the treatment lead to the development of recurrent MDR-TB disease and its transmission to three close relatives (A. Indra, Vienna, Austria. P). Presently, XDR-TB has been reported in 35 countries worldwide, among them Georgia, where it is not uncommon (N. Shubladze, Tbilisi, Georgia. P; S. Vashakidze, Louisville, USA. P), and Portugal, where the major part of the capreomycin resistance isolates contain mutations in the *tlyA* gene (J. Perdigao, Lisboa, Portugal. P) that codes for a rRNA methyltransferase (Johansen et al. 2006).

In Europe the majority of the MDR strains belong to the so-called Beijing isolates. While most of the Beijing isolates found in Asia belong to the sensitive class, the European Beijing strains are frequently resistant belonging to the MDR group. Beijing isolates are a minority of the tuberculosis strains in Europe, but they account for the majority of the resistant and more transmissible MDR-TB strains in the continent. However, in Spain most of the 171 MDR-TB isolates diagnosed from 1998 to 2007 within the population of immigrants belonged to Euro-American lineages while 14% were of the Beijing type (P. Gavin, Zaragoza, Spain. P). According to the RFLP fingerprinting,

Box 1 Tuberculosis treatment and antibiotic resistance

Treatment for TB is usually initiated with isoniazid, an inhibitor of the synthesis of mycolic acid, ethambutol, inhibitor of the synthesis of arabinogalactan and arabinomannan, that together block the synthesis of the mycobacterial mycolic acid–arabinogalactan–peptidoglycan complex, pyrazinamide, an inhibitor of the fatty acid synthetase of *M. tuberculosis*, and rifampicin, that inhibits RNA polymerase. The combined treatment extends for 2–4 months and is followed by two additional months of rifampicin and isoniazid treatment. Patient compliance is essential during the full treatment. The TB isolates that are resistant to the first-line drugs rifampicin and isoniazid are called Multi Drug-Resistant *M. tuberculosis* (MDR-TB), their treatment is more difficult and may last for longer periods, of up to 2 years, with second-line drugs that include one fluoroquinolone (gyrase inhibitor) and one injectable drug among amikacin, kanamycin, or capreomycin (inhibitors of protein synthesis). In any case the prognosis for MDR-TB is not very favorable and many cases prove fatal even if treated. Although less frequent, there are MDR isolates that are additionally resistant to fluoroquinolones and to one of the injectable antibiotics, they are called Extensively Drug-Resistant *M. tuberculosis* (XDR-TB). While new drugs against XDR-TB are under development, its treatment is difficult requiring a combination of different drugs for several years.

the Beijing genotype of *M. tuberculosis* has two main variants: there is the atypical and less frequent one that correlates to the common ancestor; and also the typical pattern, that has an apparently selective advantage over other TB strains, putatively due to point mutations in some mutator genes. Correlation between the use of BCG vaccination and antibiotic use with the prevalence of resistant typical Beijing isolates has been observed in epidemiological typing studies in Vietnam. In this country BCG vaccination may have worked by lowering the atypical Beijing type infection, while on the other hand it may have favored the selection of the typical type (D. van Soolingen, Bilthoven, The Netherlands. P).

Massive parallel sequencing of the genomes of some Beijing strains has been done and the results have been used to compare with the genomes of susceptible isolates. The data from the resistant isolates obtained from countries belonging to the former Soviet Union indicate a high degree of clustering and a remarkable ability of transmission and clonal expansion. In particular one specific cluster, with a supertransmissible phenotype, is responsible for almost one-third of the MDR cases registered in the Aral Sea region (S. Niemann, Borstel, Germany. P). In contrast, the emergence of MDR is not associated with clustering in Bulgaria (V. Valcheva, Sofia, Bulgaria. P). The detailed molecular basis underlying these high rates of transmission and expansion, and those accounting for their differences, are not fully understood, but they are suspected to be a consequence of specific point mutations that counteract the loss of fitness caused by the acquisition of resistances (as has been already documented for tuberculosis and other pathogens, Bottger et al. 2005; Andersson 2003), which makes them potentially very threatening.

The molecular characterization of different *M. tuberculosis* strains isolated in the Balkans shows a high diversity in their distribution in the region, a European cross-road area with moderate incidence of tuberculosis (C. Sola, Paris, France. P). Transmission of *M. tuberculosis* was monitored in the region indicating that MDR strains are being actively transmitted (J. Perdigão, Lisboa,

Portugal. P). Surprisingly, while in most of Europe these MDR strains are usually associated to the Beijing lineage (P. Supply, Lille, France. P), the Beijing phenotype is notoriously absent in Bulgaria where many resistant strains belong to a different specific spoligotype, ST53 (V. Valcheva, Sofia, Bulgaria. P). Differences in the frequency of antibiotic resistant tuberculosis isolates, besides having a clear geographical component, may also vary accordingly to age, as evidenced by the higher proportion of drug resistant tuberculosis reported to occur in the cases affecting children relative to the frequency observed in the adult population in Northern Buenos Aires (N. Morcillo, Buenos Aires, Argentina. P).

Genome evolution in mycobacteria

Sebastien Gagneux (London, UK. S) made an extensive review of TB phylogeography discussing the implications for drug discovery and vaccine design. Due to the virtual absence of horizontal gene transfer in *M. tuberculosis*, large sequence polymorphisms (LSPs) could be used as markers to construct a robust phylogeny which revealed six main lineages distributed in geographically distinct areas (Gagneux et al. 2006). Multilocus sequence analysis confirmed the distribution into “ancestral” and “modern” lineages and led to the “out-of-and-back-to-Africa” hypothesis about *M. tuberculosis* spreading. According to Gagneux, correlation of genetic versus geographic distance of lineages can even reproduce the different modes of distribution, which was slow by land route for the ancient lineages and quicker by modern means of transport for the modern lineage (Hershberg et al. 2008).

Analysis of the rate of non-synonymous (dN) and synonymous (dS) nucleotide substitutions in single nucleotide polymorphisms (SNPs) revealed a relatively high ratio dN/dS of 0.57 for *M. tuberculosis* which suggests that stabilizing selection is only weakly acting. This led to the conclusion that genetic diversity of *M. tuberculosis* is likely to be functional and might have phenotypic consequences with an impact on disease, which is especially relevant in the light of lineage-specific association with

particular human populations, i.e. the occurrence of separate strain lineages in distinct regions of the world.

Genetic variation may manifest itself in immunologic diversity among *M. tuberculosis* strains. Robert Wallis reported differential, strain specific, human antimycobacterial immunity toward several tester strains and suggested that immunologic diversity might account for incidences of re-infection of immunologically normal persons in high prevalence regions (R. Wallis, Washington DC, USA. S).

Host genetics

Eileen Hoal gave an overview about host susceptibility to tuberculosis (E. Hoal, Tygerberg, South Africa. S). Host genetic factors play a major role in determining differential susceptibility to TB and several relevant genetic loci have been identified by a variety of approaches. These methods include case-control studies assessing biologically plausible candidate genes, genome-wide linkage analysis of affected sibling pairs and assessment of the human homologues of susceptibility genes identified in murine models. Hoal reviewed the contribution of known loci, as Vitamin D receptor (VDR) gene variants have been shown to regulate immune response in tuberculosis (Babb et al. 2007b), and linkages found to markers on chromosomes 6, 15, 17 (Möller et al. 2009) and *X*. However, a previously postulated influence of Sp110 on tuberculosis in human adults (Tosh et al. 2006) could not be reproduced (Babb et al. 2007a). Two novel putative loci for TB susceptibility were presented: in a genome-wide approach it was found that variations in the melanocortin receptor MC3R and cathepsin Z on chromosome 20 are important for susceptibility to tuberculosis in African populations (Cooke et al. 2008). Both are related to macrophage function and are potential therapeutic targets. Hoal suggested that implementation of admixture mapping (Box 2) is a valuable complementary method for the identification of new susceptibility genes. Other work on the identification of genetic variants that favor BCG-mediated positive immune response was presented which may lead to future improvement of the BCG vaccine.

Genetics of drug resistance in MDR and XDR-TB

The association between a given genetic background, that is, the distribution of mutations in the different target genes associated with drug resistance (e.g. *rpoB* in the case of rifampicin) is well documented within the Beijing genotype (Glynn et al. 2002; Mokrousov et al. 2006; Lipin et al. 2007) and can be associated to the different *M. tuberculosis* phylogeny lineages (T. Brown, London, UK. P; H. Merdassi, Tunis-Belvedere, Tunisia. P; P. Miotto, Milan, Italy. P; M. I. Murcia, Bogotá, Colombia. P). Resistance to

streptomycin, a classical antibiotic to treat TB, is not necessarily associated with mutations in the ribosomal proteins (*rpsL* or *rrs* genes), as mutations in a rRNA methyltransferase (*gidB*) that may be responsible for low level resistance to streptomycin have been recently identified (J. Perdigão, Lisboa, Portugal. P). Besides inactivation of *katG*, resistance to isoniazid may be due to mutations in the *inhA* gene and it appears that some of them, namely those in the regulatory region, besides causing low level resistance to isoniazid, may also confer low level resistance to ethionamide, another prodrug active as well in blocking the synthesis of mycolic acid (C. Abe, Tokyo, Japan. P).

Due to the rising problem of MDR- and XDR-TB, the emergence of resistance to different second-line drugs is receiving increasing attention. A study to detect and identify the isolates resistant to second-line drugs in the Netherlands indicates that while strains resistant to second-line drugs are not frequent in Dutch patients, they are more frequently present in immigrants, in which resistances to specific antibiotics can be traced to their region of origin. This observation provides an initial clue, based on patients' provenance, to initially prescribe the most adequate therapy (J. van Ingen, Bilthoven, The Netherlands. P; van Ingen et al. 2008). As in the case of resistance to first-line drugs, the genetic identification of mutations conferring resistance to second-line drugs may lead to a quicker and more accurate diagnostic, and therefore to an improved treatment of XDR-TB. Studies conducted in isolates from Karakalpakstan, a high-incidence region in Uzbekistan, have identified hotspots in *gyrA* that confer resistance to ofloxacin and two new ofloxacin-resistance mutations located in *gyrB*. Two hotspots in the *rss* gene appear as responsible for the majority of amikacin and capreomycin resistance in this region, while a new aminoglycoside resistance-mutation was identified in the *tlyA* gene (S. Schmidt, Borstel, Germany. P). In some cases the identification of the mutation associated with resistance is not easy, as is the case in several clinical isolates of *M. tuberculosis* resistant to linezolid, in which the mutation in the *rrl* gene (23S rRNA) expected from the *Staphylococcus aureus* and *Enterococcus* linezolid-resistant mutants could not be found (no abstract, Hillemann et al. P).

Traditional and novel approaches in the diagnosis of TB and the identification of drug resistance

The highest burden of tuberculosis is found in developing countries, where the standard procedures of passive case-finding using smear microscopy have failed to control the disease. It is therefore perceived that more effective protocols are required to improve diagnosis (Box 2). Optimization of the procedures to recover bacilli may help to

Box 2 Molecular tools for diagnosis and typing

Besides technical advances assisting in the early detection of *M. tuberculosis* growth by replacing solid cultivation by liquid media that can be safely handled in largely automated self-contained instruments, molecular techniques have deeply improved the diagnosis and typing of mycobacteria, both for strain identification, genotyping and drug susceptibility testing.

MIRU-VNTR is a genotyping procedure used to distinguish between different strains of the *M. tuberculosis* complex (MTC) based on the analysis by PCR amplification of the variable number of tandem repeats (VNTR) of mycobacterial interspersed repetitive units (MIRU). MIRUs, present in the intergenic regions of several loci (in optimized protocols up to 24, Supply et al. 2006), can be distinguished by their different sizes (in the range of 50–100 bp). Their identity and number is then compared to a MTC database using computational tools that can be implemented on-line (<http://www.miru-vntrplus.org>). MIRU-VNTR typing allows high-throughput, discriminatory and reproducible analysis of clinical isolates, and to some extent may tend to replace the more cumbersome IS6110 RFLP fingerprinting procedures (Cave et al. 1991).

Spoligotyping, spacer oligonucleotide typing, one of the molecular tools initially used for detection and genotyping of *M. tuberculosis*, is a procedure based on the PCR amplification of the highly polymorphic direct repeat (DR) locus of the mycobacterial chromosome (Kamerbeek et al. 1997). The MTC DR locus is one of the most well studied loci showing considerable strain-to-strain polymorphism (Fang et al. 1998). It contains several 36 bp repeats interspersed by non-repetitive DNA 35–41 bp spacers. The spoligotyping procedure has maintained its place in the molecular epidemiology of TB, as it allows a quick identification of the main families within the MTC.

Line-probe assays, as LiPA and Hain test, using PCR amplification of *M. tuberculosis* DNA are widely used and are commercially available to detect TB as well as multi-drug resistant MDR-TB. The amplified material in these assays is conveniently visualized on a strip by the presence or absence of bands.

Admixture mapping, a whole-genome approach procedure able to resolve ethnic differences in the inheritance of genes in populations of mixed ancestry (McKeigue 1998), can be used to localize disease-susceptibility genes (Reich and Patterson 2005) and it appears as a promising tool to determine genetic susceptibility to TB in different human populations of mixed provenance (Hardy et al. 2008).

improve detection, for example the improvement of the current smear microscopy by concentration of the bacilli in sputa (S. Wilson, London, UK. P), and the immuno-capture of bacteria from water supplies (C. Kluge, Paris, France. P), both using magnetic separation, and, in the latter one, quantitative PCR to amplify a *Mycobacterium* housekeeping gene, make it possible to detect as few as 50 copies of the genome per liter. Technologies based on quantitative Real-Time PCR, help in the detection of bacilli by increasing the sensitivity of the reaction in specific samples either from clinical origin (M. Chomarat, Pierre-Benite, France. P; H. Simsek, Ankara, Turkey. P) or dairy products (J. Donaghy, Belfast, N. Ireland. P). These procedures, applicable only in developed countries, have the advantage of helping for a correct diagnosis in difficult samples, such as the paucibacillary samples in extrapulmonary specimens (E. Fernandes, Lisboa, Portugal. P). Other advances that may allow a more efficient and early diagnosis are being evaluated, including immunodetection methods such as the use of lipoarabinomannan antigen to detect bacilli in urine and sputum (R. Mutetwa, Harare, Zimbabwe. P) and a new bio-optical device allowing rapid detection of *M. tuberculosis* antigens in the patient's breath (R. Mc Nerney, London, UK. P), as well as a novel procedure using intrinsic auto-fluorescence of *M. tuberculosis* allowing its detection without staining (L. Salazar, Caracas, Venezuela. P; Patiño et al. 2008).

While an effective control of tuberculosis strongly relies on the establishment of a correct treatment, prescription of the treatment depends on an early and accurate identification of the drug resistance/susceptibility of the isolates. As

MDR- and XDR-TB are now a very serious threat for the effective control of tuberculosis, there is a pressing need for a rapid and accurate detection of the bacterial drug-response. Detection of drug resistance can now be accurately and rapidly made with commercially available diagnostic tools, largely automated, based on the detection of bacterial growth in liquid media. However, these procedures are usually expensive and require well-trained professionals, conditions that hamper their use when resources are low and training is deficient. Colorimetric assays based on redox indicators and nitrate reductase activity, microscopic examination of microcolonies and phage technology, although more cumbersome, have proven useful in these low-resource settings. (J. C. Palomino Antwerp, Belgium. P; Palomino et al. 2008). Once the mutation associated with drug resistance in a particular target gene is known, drug resistance/susceptibility can be determined using molecular approaches. One of the well-established molecular methods based on amplifying DNA present in the sputum by polymerase chain reaction, the Line Probe Assay (LiPA), Box 2, allows the rapid detection of MDR isolates. The line-probe assay is currently recommended by the WHO for an early and quick detection of MDR bacilli, and therefore has been widely applied for that purpose. The studies conducted in Russia, Spain, Greece, Portugal and Latvia show a good correlation between the results obtained with the LiPA protocol and those found using bacterial growth tests to measure resistance (V. Nikolayevskyy, London, UK. P; M. Causse, Cordoba, Spain. P; P. Ioannidis, Athens, Greece. P; R. Macedo, Lisboa, Portugal. P; G. Skenders, Riga, Latvia. P).

Molecular and cell biology

Mycobacterium/macrophage interaction

The pathogenic lifestyle of *M. tuberculosis* implies infection of host professional phagocytic cells. In parasitized macrophages the bacterium resides inside a modified phagosome which promotes intracellular growth and helps to escape detection by the immune system.

Using cryo-immunogold electron microscopy, Peter Peters showed that *M. tuberculosis* can disrupt the phagosomal membrane and enter the cytosol 4 days after infection (P. Peters, Amsterdam, The Netherlands. S). Maria Lerm confirmed this finding using high resolution transmission electron microscopy and confocal microscopy. She demonstrated that during prolonged infections a fraction of the mycobacterial population exits into the cytoplasm, while another fraction resides in mature phagosomes (M. Lerm, Linköping, Sweden. S). Moreover, Peter Peters reported that the ability to evade from the phagosome is dependent on the ESX-1 secretion system (see below). In fact *M. bovis* BCG or an *esx-1* mutant of *M. tuberculosis* reside inside the phagosomes even 4 days after the infection (van der Wel et al. 2007). Maximiliano G. Gutiérrez challenged these findings, reporting electron microscopy experiments in which the translocation of *M. tuberculosis* to the cytoplasm was not evidenced (M. Gutiérrez, Heidelberg, Germany. S). Although different experimental conditions and cell lines used in these laboratories could account for the differences, future work should dispel this controversy and elucidate the role of phagosomal escape in the pathogenic cycle.

New data about *M. tuberculosis* strategies to avoid killing by macrophages were reported by Yossef Av Gay (Vancouver, Canada. S). Dephosphorylation of the human Vacuolar Protein Sorting 33B (VPS33B) by the secreted mycobacterial tyrosine phosphatase PtpA mediates *M. tuberculosis* survival within macrophages inhibiting phagosome–lysosome fusion. This supports the idea that *M. tuberculosis* has developed strategies to specifically interfere with the complex regulatory pathway involved in phagosome maturation (Bach et al. 2008).

Amanda Welin reported the importance of incorporation of *M. tuberculosis* lipoarabinomannan (LAM), a prominent cell wall component, into macrophage membrane rafts as a prerequisite for phagosomal maturation block (A. Welin, Linköping, Sweden. S). This underscores the complexity of the relationship between *M. tuberculosis* and its host (Welin et al. 2008). Using an in vitro method mimicking LAM shedding from the mycobacterial surface upon infection, she demonstrated that LAM is incorporated into membrane rafts via its glycosylphosphatidylinositol (GPI) anchor. The incorporation of ManLAM (mannose-capped

LAM found in *M. tuberculosis*), but not PILAM (phospho-*myo*-inositol capped LAM, found in avirulent mycobacterial species), resulted in reduced phagosomal maturation. However, during in vivo pathogen–macrophage interaction the role of ManLAM may not be determining, since mycobacterial mutants lacking the mannose cap of LAM are not necessarily impaired for intracellular survival (Appelmeik et al. 2008).

Lalita Ramakrishnan reviewed the *M. marinum*-zebrafish model (L. Ramakrishnan, Seattle WA, USA. S). This pathogenic mycobacterium shares several pathogenicity mechanisms with *M. tuberculosis*, but it is a fast grower, and does not require a BLS-3 facility for its manipulation (Tobin and Ramakrishnan 2008). The zebrafish model has two major advantages: the first is the possibility to investigate mycobacterium–host interactions in the sole context of innate immunity (since the adaptive immunity develops only after the third week of embryo life), the second is its optical transparency (Lesley and Ramakrishnan 2008). Using this model it was possible to follow granuloma formation in vivo and discover that these are not impermeable structures as previously thought. Moreover, it was possible to demonstrate that the ESX-1 locus is essential for the formation of granuloma during the first phases of infection. The finding that granuloma formation is promoted by a bacterial virulence determinant suggests the interesting hypothesis that these structures may not only represent a host–protective response. Rather, they could be involved in the bacterial multiplication and spread, at least during the early phase of infection (Davis and Ramakrishnan 2009).

Type VII secretion systems

The type VII secretion systems (or T7SS) of *Mycobacterium tuberculosis* (Abdallah et al. 2007) are specialized for the secretion of proteins that lack a canonical signal sequence, among them the highly immunogenic proteins ESAT-6 (early secreted antigenic target of 6 kDa) and CFP-10 (culture filtrate protein of 10 kDa). Roland Brosch gave an overview of the current knowledge about the role of ESAT-6 and CFP-10 in attenuation and pathogenicity (R. Brosch, Paris, France. S). The respective genes are located within the *esx-1* region, encoding one of the five type VII secretion systems (ESX-1–ESX-5) present in the *M. tuberculosis* genome (Abdallah et al. 2007). ESAT-6 and CFP-10 form a heterodimer in the cytoplasm which is recognized by the secretion system through a signal sequence present in the CFP-10 C-terminus (Renshaw et al. 2005). Both *M. microti* and *M. bovis* BCG contain a deletion in the *esx-1* region which is one of the main causes of their attenuation suggesting that these two proteins have an important role in pathogenicity (Brodin et al. 2006;

Gordon et al. 1999). To reinforce this hypothesis, it was recently demonstrated that a strongly attenuated strain of *M. tuberculosis*, H37Ra, is unable to secrete ESAT-6 because of the down-regulation of the genes *rv3614c–rv3616c* (*espA*). This gene cluster is essential for ESAT-6 and CFP-10 secretion (Frigui et al. 2008). Down-regulation of *rv3614c–rv3616c* was shown to be due to a C to T mutation responsible for a serine to leucine replacement at position 219 of the two-component regulator PhoP, a protein well known for its involvement in *M. tuberculosis* pathogenicity. Interestingly, a well characterized attenuated *M. tuberculosis* *phoP* deletion mutant is able to induce a strong protective immune response and is one of the best available candidates for the development of a new vaccine against tuberculosis (Asensio et al. 2008, see below).

The biological function of the ESAT-6 and CFP-10 heterodimer is not yet completely understood, but the finding that at low pH they can dissociate from each other, allowing ESAT-6 to interact with liposomes suggests an interaction of this protein with the phagosomal membrane after phagosomal acidification (de Jonge et al. 2007).

The importance of type VII secretion systems in mycobacterial physiology was underscored by Kari Ann Sweeney (Bronx, USA. S), which reported that ESX-3 is essential in *M. tuberculosis*, but not in *M. smegmatis*. However, *M. smegmatis* mutants lacking this gene cluster were severely attenuated in mice. Attenuation was due to inability to evade both innate and adaptive immunity. Surprisingly, mice infected with *M. smegmatis* *esx-3* mutants showed a marked increase of survival and bactericidal killing over time upon challenge with *M. tuberculosis*. This was characterized by a strong Th1 response, which opens new opportunities for vaccine development.

Finally, Wilbert Bitter, Daria Bottai, and Mariagrazia Di Luca, reported in different contributions (W. Bitter, Amsterdam, The Netherlands. S; D. Bottai and M. Di Luca, Pisa, Italy. S), the construction of *esx-5* mutants in *M. marinum* and *M. tuberculosis* underlining its importance for growth in macrophages, immunomodulation, and secretion of the members of two peculiar mycobacterial protein families: PE_PGRS and PPE (Abdallah et al. 2007). The names PE and PPE are derived from the motifs Pro-Glu (PE) and Pro-Pro-Glu (PPE) and proteins of the PE_PGRS subfamily consist of the PE domain followed by a C-terminal extension with multiple tandem repetitions of Gly-Gly-Ala or Gly-Gly-Asn encoded by the PGRS motif [polymorphic GC-rich repetitive sequences] (Banu et al. 2002). Alessandro Cascioferro reported that the PE domain of PE_PGRS33 is essential for the export of this protein to the mycobacterial outer membrane (A. Cascioferro, Padua, Italy. S; Cascioferro et al. 2007). In the future it will be interesting to study the interactions between this PE domain and the components of the ESX-5 secretion system.

New insight on mycobacterial surface

Both David Minnikin and Mamadou Daffé presented new models of the mycobacterial cell wall based on recent chemical and ultrastructural findings (D. Minnikin, Birmingham, UK. S; M. Daffé, Toulouse, France. S). Daffé investigated the cell envelope of *M. bovis* BCG, *M. smegmatis* and *Corynebacterium glutamicum* in a native state by cryo-electron microscopy of vitreous sections (Zuber et al. 2008). This work visualized directly the presence of a bilayer outer membrane (OM), a structure that has been postulated for a long time for mycobacteria from conventional studies, and a periplasmic space in all three species. Moreover, it was shown for a *C. glutamicum* Δ *pks13* mutant which lacks mycolic acids that these are indispensable constituents of the OM structure. Recently, similar results have also been reported by Hoffmann et al. (2008) who revealed the OM by cryo-electron microscopy of vitreous sections and cryo-electron tomography of intact cells. However, albeit both studies came to similar results with regard to the bilayer nature of the OM, their models of how exactly the arrangement of the lipids accommodate for its thickness, which is thinner than expected, differ. Zuber et al. propose a zipper model in which the hydrocarbon chains of the lipids of both OM layers are not only present in a compact, folded conformation, as suggested by studies with artificial Langmuir monolayers (Villeneuve et al. 2007), they also propose that the arabinogalactan-bound mycolic acids in the inner leaflet have to intercalate to a certain extent with the chains of free lipids from the outer OM leaflet.

The confirmation of the presence of an outer membrane is extremely important for the understanding of mycobacterial physiology and host interaction. New insights into the fine structure of the mycobacterial envelope, as for example the finding that LAM is not exposed on the surface of in vitro-grown cells (Alsteens et al. 2008), raise questions for further investigations of whether and how this important structure may be modified within host environments.

New tools

Adrie Steyn presented tools for the investigation of protein–protein interactions in *Mycobacteria* and gave examples for their successful application (A. Steyn, Birmingham AL, USA. S). To overcome the limitations of the heterologous yeast-two-hybrid system, the mycobacterial protein fragment complementation system was designed to detect protein–protein interactions in *M. smegmatis* (Singh et al. 2006). The system is based on the reconstitution of a functional dehydrofolate reductase whose different domains are fused to the proteins of interest and allow, in case of interaction of these proteins, the growth of

M. smegmatis on trimethoprim. The additional ability to quantify the strength of protein interactions with an Alamar-blue redox assay was presented as an advantage of mycobacterial protein fragment complementation. In order to make the system more versatile, new derivatives including GATEWAY vectors and the introduction of a tetracycline-inducible promoter have been developed. A similar system is the “Split-Trp” sensor system which is suitable for detection of protein interactions in *Escherichia coli* and *M. smegmatis* (O’Hare et al. 2008).

Francesca Boldrin reported the construction of a repressible promoter system based on the Tn10-derived tetracycline repressor TetR and *Streptomyces pristinaespiralis* pristinamycin repressor Pip (F. Boldrin, Padua, Italy. S). The gene of interest is placed under transcriptional control of a Pip-dependent promoter, while the Pip-encoding gene is transcribed from a TetR-repressible promoter. The addition of tetracycline results in the induction of Pip transcription and, as a direct consequence, in the transcriptional repression of the gene of interest. The system was successfully used to construct a *ftsZ* conditional mutant in *M. smegmatis*, and a *fadD32* conditional mutant in *M. tuberculosis*.

John McKinney investigated the phenomenon of phenotypic resistance against antibiotics in mycobacterial cultures at the single cell level (J. McKinney, Lausanne, Switzerland. S). For this purpose bacteria were grown in a microfluidic device and their behavior monitored by automated time-lapse fluorescence microscopy. This set-up allowed the observation of the growth dynamics of GFP-expressing *M. smegmatis* cells under changing culture conditions, including exposure to antibacterial drugs. Two different sub-populations of bacteria were identified, one which was killed by isoniazid and one which was able to survive in a slowly-proliferating stage. This assay can be scaled up for high throughput screening and allows for continuous observation of the same individual cells for up to 2 months. Thus, it will be possible to screen libraries of *M. tuberculosis* mutants to identify the genes responsible for the capability of these bacteria to survive drug treatment. This new technique will certainly open new perspectives for understanding the mechanism of persistence and for developing strategies to improve the efficacy of antibacterials.

The establishment and properties of latency

Latency being the most frequent and lasting state of the tuberculosis infection, the accurate diagnosis as well as the adequate treatment of latent tuberculosis infection is a pressing need. Diagnosis of latent infection using interferon gamma release assays is nowadays preferred in the

developed nations over the tuberculin test for the advantageous detection of infections occurring in BCG-vaccinated individuals. However the interferon release assay, although useful, presents some difficulties when it is used at locations with limited resources and high prevalence of HIV (P. de Haas, Lusaka, Zambia and London, UK. P).

Most antimycobacterial drugs are useful against actively dividing bacilli, therefore they are not so useful in the treatment of latent infection, as bacteria are supposed to be under a dormant stage. Lanfranco Fattorini described that a combination of drugs (rifampin, metronidazole, moxifloxacin or amikacin) are active in the Wayne model system (Wayne and Hayes 1996) against aerobic and anaerobic non-proliferating bacilli. Those conditions are commonly accepted to reproduce the hypoxic conditions of latency. The combination of drugs may be useful for the clearance of dormant bacteria present in latent infections (L. Fattorini, Rome, Italy. P; Iona et al. 2007).

The pathogenic basis of the latent infection is still controversial, as little is known on how the bacteria can evade the immune system. Recently, a dynamic hypothesis of latent infection has been proposed based on the cellular turnover in lungs and the fact that isoniazid treatment, an antibiotic that should not be effective against resting bacteria, has demonstrated to be active to clear “dormant” bacilli during the latent infection (P. J. Cardona, Badalona-Catalonia, Spain. P; Cáceres et al. 2009). It can be hypothesized that non-proliferating bacilli are drained out of the granuloma by foamy macrophages but a constant endogenous reinfection could occur, thus maintaining the latent infection (Peyron et al. 2008). This observation is being exploited to obtain a non-living vaccine that is expected to prevent the reactivation of the disease.

During latency, the bacilli are considered to undergo a non-proliferative stage with low metabolic activity and a diminished rate of division, however this arrest of bacterial cell division does not result in a loss of long-term viability. The control mechanisms that couple cell growth, cell division and viability in latent mycobacteria are poorly known, the development of tools to obtain insights into the cytolocalization of the proteins involved in this processes, such as FtsZ, FtsQ or DivIVA, being therefore of much interest (M. Vicente, Madrid, Spain. P). Using these kind of tools, the in vivo interaction between ParB, ParA and DivIVA (wag31), all of them proteins participating in the partition of the bacterial chromosome during cell division, was shown (Casart et al. 2008). Specifically, ParB is localized in the cell poles (Y. Casart, Caracas, Venezuela. P) exhibiting a localization pattern compatible with a role in *oriC* segregation.

Some experimental data indicate the possibility that host cells other than macrophages participate in the pathogenicity of mycobacterial infections (García-Pérez et al.

2008) and it was discussed how *M. tuberculosis* can then avoid the usual immune response of the infected host. J. Luna-Herrera (outside the programme. P) discussed how nonphagocytic cells may play a role in tuberculosis and showed that B cells are able to internalize *M. tuberculosis* and *M. smegmatis* using a macropinocytosis entrance mechanism. Contrary to *M. smegmatis*, *M. tuberculosis* subsequently survives inside these infected B cells, possibly due to its failure to induce sustained levels of nitric oxide production.

A new finding that may revolutionize our understanding of the role of non-growing bacilli during the infection cycle was presented by Simon Waddell (London, UK. S). By RNA profiling of *M. tuberculosis* cells from human sputa, he revealed that bacilli released from cavitating lesions are not rapidly proliferating as previously suggested. Rather, transcriptional signatures suggest that an important population of bacilli in sputum exists in a slow/non-proliferating state, which might help them to survive adverse conditions during transmission (Garton et al. 2008). Future research is needed to examine the clinical implications of this finding and its consequences for new *M. tuberculosis* drug development strategies.

From genome to vaccine and drug discovery

Strategies to find new tools to combat tuberculosis

The search for new anti-mycobacterial drugs is, given the alarming scenario caused by the disease, of great importance to alleviate human suffering. The current efforts in drug discovery include the screening of both natural compounds, as those found in traditional myrtle oils that show activity against a variety of MDR and XDR-TB (S. Cannas, Sassari, Italy. P) and chemically synthesised compounds as *N*-benzylthioacylthioamides or 4-(benzylsulfanyl)pyridine-2-carbothioamides that show activity against both TB and several NTMs (V. Klimešová, Hradec Králové, Czech Republic. P; Klimešová et al. 1999; Dolezal et al. 2009). New avenues to obtain active drugs may also be found through the searching of new potential targets. In this line, the *accD6* gene (Rv2247), a member of the FAS II operon coding for acetyl-CoA carboxylase β subunit that plays a crucial role in the synthesis of mycolic acid, is essential in *M. tuberculosis*, as shown by the impossibility of deleting it when present in single copy (J. Pawelczyk, Lodz, Poland. P). Surprisingly the ortholog gene is not essential in *M. smegmatis*, although the deleted strains show a significant decrease in viability (J. Pawelczyk, Lodz, Poland. P).

Stewart Cole presented advances in the effort of the of the European “New medicines for Tuberculosis (NM4

TB)”—Consortium to find new antibiotics against TB (S. Cole, Lausanne, Switzerland. S). Cole pointed to the additional difficulty that any new drug should ideally be compatible with other anti-tuberculosis compounds since treatment of TB likely will be only possible with a combinatory treatment. The strategy presented was dual, with the attempt to optimize hits for carefully selected mycobacterial target molecules on the one hand, and a whole cell MIC and compound-based approach to generate leads on the other hand.

To target the metabolic capacity of *M. tuberculosis*, glutamine synthetase (GS) GlnA1, which plays an important role in nitrogen assimilation, was chosen. In contrast to the other three GSs, GlnA1 is an essential enzyme for growth of *M. tuberculosis* in vitro and in vitro (Tullius et al. 2003). L-Methionine-sulfoximine (MSO) is a known and effective inhibitor of GS that was also shown to inhibit growth of *M. tuberculosis* in vitro and in vitro (Harth and Horwitz 2003). However, due to its epileptogenic properties MSO cannot be used directly as an antitubercular drug, rather, it served as a template for the design of further developable derivatives. Unfortunately, a first high throughput screen has to be considered a failure since whole cell MICs did not reflect the effective enzymatic inhibition (biochemical IC_{50}) by MSO and the inhibitory effect of several compounds was reversible by external addition of glutamine.

Targeting mycobacterial signal transduction was presented as a promising approach to find new anti-tuberculous compounds. PknB is one of eleven *M. tuberculosis* eukaryotic-like Ser/Thr protein kinases which is required for growth (reviewed in Wehenkel et al. 2008). With the identification of GarA as putative physiological substrate of PknB a specific biochemical kinase assay could be developed and several inhibitors for the kinase were identified (Villarino et al. 2005; Fernandez et al. 2006; Székely et al. 2008). Current research focuses on improvement of mitoxantrone, a hit compound used in cancer therapy which docks into the adenine-binding site of the intracellular PknB kinase domain (Wehenkel et al. 2006).

A different approach to exploit PknB signaling inhibition was presented by Sophie Magnet and is based on targeting of the reiterated extracellular PASTA (for “penicillin-binding protein and serine/threonine kinase associated”) domains (S. Magnet, Lausanne, Switzerland. S). PASTA domains are thought to interact with different unlinked peptidoglycan stem peptides and this interaction in turn activates the intracellular kinase domain, thus triggering the signaling pathway that leads to peptidoglycan remodeling (Jones and Dyson 2006). The identification of competitive PASTA domain ligands that block this signal transduction could lead to compounds that interfere with essential cell wall remodeling processes.

In a MIC-driven approach which comprised also an *ex vivo* macrophage assay and the murine infection model, benzothiazinone (BTZ) compounds were found to be highly potent inhibitors with effectiveness inclusively against MDR/XDR-TB. In order to identify the cellular target of BTZ to define their mode of action, a genetic approach, aimed at the selection for BTZ resistant mutants, was presented by Giulia Manina (Pavia, Italy. S). BTZ was found to act on Rv3790, a protein involved in D-arabinose biosynthesis which is crucial for the production of arabinogalactan components of the mycobacterial cell wall (Makarov et al. 2009; reviewed in Wolucka 2008).

Reconstructing the genesis of the BCG vaccine through genomics

The vaccine strain, *M. bovis* BCG, although not fully effective in prevention, is the most extensively used vaccine in history and therefore the description of the genetic changes that occurred from the parental to the vaccine strain have received considerable attention in an attempt to gain further knowledge to be applied in developing new and more efficient vaccine strategies. BCG has suffered genomic changes since its initial isolation, mostly due to the adaptation to grow *in vitro* under stressing conditions, namely in the presence of beef bile, and being forced to use glycerol as a nutrient. A striking phenomenon is gene duplication, uncommon within members of the *M. tuberculosis* complex. Deletions and duplications have allowed the deduction of the genealogy of the current BCG strains, separating on one side BCG Pasteur from the rest of the variants. The role of some deletions (particularly RD1, causing the loss of the protein secretion system ESX-1) in attenuation has been investigated and seems clear (Mahariras et al. 1996; Pym et al. 2002). On the other hand, one of the duplications, DU1, affecting 29,667 bp and found exclusively in the Pasteur BCG variant, contains a duplication of *oriC*. This latter duplication may cause problems in chromosome replication, which could decrease fitness. Another duplication, DU2, affecting 39 Kbp is found in four different forms among the BCG derivatives. The variation seen in DU2 is more complex than that of DU1, as it contains a small internal deletion within one of the two copies and, as noted, it is heterogeneous in the different BCG strains. The functional implications are hard to define, but it may play a role in glycerol metabolism, the carbon source used by the Calmette-Guérin bacillus (S. Gordon, Dublin, Ireland. P). Functional analysis of single nucleotide polymorphisms (SNPs) comparing *M. bovis* and BCG could help to explain some of the phenotypic differences between these respectively virulent or avirulent strains. One of the problems faced by this research is that the original *M. bovis* strain subcultured by

the Institute Pasteur scientists at the beginning of the twentieth century was lost. Hence, to identify putative *in vitro*-derived mutations, comparisons have been made with existing UK and French *M. bovis* isolates in the hope of finding attenuating mutations. Among the genes carrying SNP differences between *M. bovis* and BCG is the *pykA* gene, encoding pyruvate kinase. This gene is inactive in wild type *M. bovis*, while it has regained activity in BCG due to a point mutation reversion. This most likely is due to the pressure applied to BCG for growth in media containing glycerol. The mutation in *M. bovis pykA* not only means that it requires pyruvate to be added to media *in vitro*, but also means that it must rely on fatty acids or aminoacids as its *in vivo* energy source (Keating et al. 2005). However the *in vitro* culturing of BCG bacilli clearly selects for strains in which glycerol metabolism is enhanced; for example amplification of the *glpD2* gene, encoding glycerol-3-phosphate dehydrogenase, is observed in DU2-I and DU2-IV, thus leading to a 2.7-fold increase in expression levels in these BCG strains compared with *M. bovis* (Brosch et al. 2007).

Additional differences deduced from the identified SNPs that may have effects on the virulence of the strain affect *phoP*, mutation of which in *M. tuberculosis* produces alterations in the cell envelope and attenuation (see below, Gonzalo-Asensio et al. 2006; Pérez et al. 2001), and *sigK* which effects the expression of the genes encoding the major antigens MPB70 and MPB83 (Charlet et al. 2005).

Replacing the BCG vaccine (new vaccine strains)

Carlos Martín presented advances in the development of a rationally attenuated vaccine strain based on the *M. tuberculosis phoP* mutant (C. Martín, Zaragoza, Spain. S). PhoP has been identified as an important virulence factor (Pérez et al. 2001). A major tuberculosis disease outbreak with a high mortality rate between HIV patients was shown to be caused by a *M. bovis* B strain which overexpressed this gene (Rivero et al. 2001; Soto et al. 2004). An attenuated live vaccine, as the one proposed by Martín, is likely to have a better protective performance than *M. bovis* BCG since it conserves several main immunodominant antigens which are missing or downregulated in BCG due to extensive deletions (Martín et al. 2006; Reece and Kaufmann 2008). However, a main point of concern with recombinant live vaccines is the question of safety. Therefore, to enter phase I clinical trials, the Geneva consensus established several requirements (Kamath et al. 2005). Two of them are the introduction of an independent second non-reverting, preferably unmarked, mutation into the vaccine candidate strains and the exigency for rigorous testing of the strain's safety profile. Following this strategy Ainhoa Arbués (Zaragoza, Spain. S) presented a promising

candidate toward the development of a *M. tuberculosis* live vaccine (Asensio et al. 2008).

Martín also reported further functional genetic analyses of *phoP*, the response regulator of the *phoP-phoR* two component signal transduction system. The central role of the regulator in *M. tuberculosis* biology was emphasized by the finding that 2% of the genes are differentially regulated in the *phoP* mutant, among them several genes encoding for the synthesis of lipids that are important for virulence (Walters et al. 2006; Gonzalo-Asensio et al. 2008a). Further genetic characterization revealed that in H37Rv *phoP* is subject to a positive transcriptional autoregulation and that *phoP-phoR* are transcribed as an operon (Gonzalo-Asensio et al. 2008b).

Non-tuberculous mycobacteria

The rising problem of non-tuberculous mycobacteria

Contrary to what was described years ago, the isolation of non-tuberculous mycobacteria (NTM) in clinical samples is more and more frequent, particularly in some populations. The number of NTM species recognized in the present has climbed to 140 from the mere 44 recorded in 1984, and it is estimated that no less than 50% are potential opportunistic pathogens. NTM with clinical relevance appear to be particularly serious in those areas in which HIV is prevalent leading to an increase in NTM cases related to AIDS (L. Rigouts, Antwerp, Belgium. P).

Geographical areas like Zambia, in which part of the population is highly isolated seem to show a higher frequency (fourfold) of NTM isolates present in the samples collected in the community relative to the tuberculosis group ones (P. Mwamba, Lusaka, Zambia. P). If proven correct, this may be a cause of concern, as the NTM are not as fully characterized as the conventional *M. tuberculosis* strains are, they contain a high frequency of antibiotic resistant isolates, and demand very specific diagnostic and therapeutic approaches. It seems necessary to confirm their presence in the clinical samples as this will have a decisive influence on the management of the patients. A PCR-based fingerprinting method already considered as being reliable for the typing of *M. tuberculosis*, the MIRU-VNTR (Box 2; C. Allix-Béguec, Lille, France. P), is now undergoing evaluation for its performance in the characterization of other mycobacteria (F. Dauchy, Bordeaux, France. P; M. Pate, Ljubljana, Slovenia. P). Drug susceptibility determination in NTM is of increasing diagnostic interest as, in the case of co-infection, these mycobacteria, usually showing a high drug resistance, can interfere with the identification of *M. tuberculosis* (L. Rigouts, Antwerp, Belgium. P). NTM

drug susceptibility was found conserved within species, clarithromycin and rifabutin being the most active drugs against them (J. van Ingen, Bilthoven, The Netherlands. P).

Among the mycobacterial strains of veterinary interest, *M. bovis* and members of the *M. avium* complex (MAC) still are those having a more relevant impact on human health (A. Zirritis, Jelgava Latvia. P; P. Moebius, Jena, Germany. P). *M. bovis*, the causative agent of tuberculosis in cattle and also a human pathogen, has a considerable relevance as a pathogen in diverse farm animals (M. Zolnir-Dovc, Golnik, Slovenia. P; J. McLernon, Kildare, Ireland. P; S. Spicic, Zagreb, Croatia. P; L. Durnez, Antwerp, Belgium. P), as well as being a potential source of contamination in dairy products (R. Forgrave, Belfast, N Ireland. P). *M. avium* subsp. *paratuberculosis* is the ethiological agent of paratuberculosis (Johne's disease), a chronic enteritis that affects mainly to domestic ruminants and causes serious economic problems. Application of molecular typing methods (RFLP, MIRU-VNTR and MLSSR) to this mycobacterium has been useful for differentiation of veterinary isolates and to perform epidemiological studies (I. Fritsch and P. Möbius, Jena, Germany. P; Möbius et al. 2008). There are other Mycobacteria in which their potential relevance has been recently investigated, for example lesions ascribable to *M. marinum* were detected at high frequency in farmed fish that, on the other hand, were largely asymptomatic (M. Prearo, Turin, Italy. P). These infective focuses are potentially threatening for humans as highlighted by the report of another case of pulmonary disease caused by *M. marinum* (H. Saito, Hiroshima, Japan. P) following the first clinical case described in 2005 (Lai et al. 2005).

Besides their relevance in human and veterinary clinic, mycobacteria not belonging to the *M. tuberculosis* complex are receiving increasing attention due to their remarkable ability to adapt to extreme environments as shown by the ability of mycobacterial isolates obtained from thermal springs in Yellowstone Park to thrive in the presence of heavy metals, high temperature or acidic media. Thus, efforts are being conducted to develop a global project seeking to identify the environmental mycobacteria present in water reservoirs in the planet (R. Santos, Lisboa, Portugal. P). The adaptation to diverse environments may involve the ability of the bacteria to form biofilms, a characteristic scarcely studied in the genus. Together with some previously described species, formation of biofilms has been observed in several clinical strains of non-pigmented rapidly growing mycobacteria, in which development of biofilm seems to correlate with the clinical significance of each isolate (N. Z. Martín-de-Hijas, Madrid, Spain. P).

Plasticity of the mycobacterial genomes

As already stated, the veterinarian relevance of *M. avium* subsp. *paratuberculosis* has urged the development of a dedicated combined array system for their study. Besides its usefulness for strain identification, the array has been used to compare the genome of the two members of the MAC whose genome-sequences are completed and annotated: *M. avium* subsp. *hominisuis* strain 104 and *M. avium* subsp. *paratuberculosis* strain k10. The comparison reveals an important degree of genomic plasticity mediated by integrases and transposases resulting in large dynamic duplications flanked by insertion sequences in *M. avium* subsp. *paratuberculosis* isolates. Activation of the transposase activity has been documented from changes in the *paratuberculosis* proteome upon infection of a human macrophage cell line (T. Bull, London, UK. P).

Genomic plasticity mediated by insertion sequences was also found to occur between two non-related mycobacterial species, *M. avium* and *M. kansasii*, most probably resulting from horizontal gene transfer occurring during coinfection of an HIV-positive patient (S. Cardoso Leão, Sao Paulo, Brazil. P). In this case, the main insertion sequence of *M. avium*, IS1245, not previously found in *M. kansasii*, was present and able to jump to several positions in some of the *M. kansasii* colonies isolated from the patient. IS1245 is able to replicate and transpose in the new genomic environment of *M. kansasii*, a species not closely related to its natural host. This finding suggests that genetic mobility may be relevant in the generation of diversity in the NTM.

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