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Tesis Doctoral

A ROLE OF EFFLUX PUMPS IN INTRINSIC DRUG RESISTANCE AND VIRULENCE OF MYCOBACTERIUM TUBERCULOSIS

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A role of efflux pumps in intrinsic drug resistance and virulence of *Mycobacterium tuberculosis*.

Memoria presentada por

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Licenciada en Bioquímica

Para optar al grado de Doctor por la Universidad de Zaragoza

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JOSÉ ANTONIO AÍNSA CLAVER, Profesor Titular de Microbiología del Departamento de Microbiología, Medicina Preventiva y Salud Pública de la Facultad de Medicina, como director de la Tesis Doctoral de **MARÍA CRISTINA VILLELLAS ARILLA**, titulada:

A role of efflux pumps in intrinsic drug resistance and virulence of *Mycobacterium tuberculosis*.
(Implicación de las bombas de eflujo en la resistencia intrínseca a fármacos y virulencia de *Mycobacterium tuberculosis)*

EXPONE:

Que esta Tesis Doctoral corresponde con el proyecto de tesis presentado y aprobado en su momento, no habiéndose producido ninguna variación.

Que dicha Tesis doctoral reúne los requisitos necesarios para optar al título de Doctor y a la mención de Doctorado Europeo.

Por lo anterior, emito el presente **INFORME FAVORABLE**.

Zaragoza, 10 de Diciembre de 2012.

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RESUMEN

Las bombas de eflujo son proteínas de membrana que transportan distintos compuestos al exterior de la bacteria. Se ha descrito que están implicadas en resistencias intrínseca y adquirida a varias drogas, así como en colonización y persistencia de bacterias patógenas en el hospedador. Además, diversos procesos fisiológicos pueden verse afectados por estas proteínas de membrana, con dependencia de cuál sea el sustrato natural de la bomba de eflujo.

En la actualidad, las cepas de *Mycobacterium tuberculosis* resistentes a antibióticos suponen un grave problema de salud. No solo se han descrito cepas multi y extensivamente resistentes, si no también resistentes a todos los antibióticos testados. Por ello es necesario un esfuerzo a nivel global que permita controlar esta situación de emergencia. Algunas de las estrategias seguidas son: el desarrollo de nuevos antibióticos contra la tuberculosis y el programa DOTS (Directly Observed Treatment Short) de la OMS. Un mejor conocimiento de los mecanismos de resistencia a antibióticos nos proporcionaría buenas herramientas para mejorar el tratamiento antituberculosis.

Las bombas de eflujo MFS Rv1258c (Tap), Rv2333 (Stp) and Rv1410c (P55) de *M. tuberculosis* se habían estudiado previamente en el organismo heterólogo *M. bovis* BCG, la cepa vacunal. Se observó como resultado que éstas estaban implicadas en la resistencia intrínseca a varios compuestos. Aunque el modelo de *M. bovis* BCG es una buena aproximación, debido al alto porcentaje de identidad entre *M. tuberculosis* y M. *bovis BCG*, las pequeñas diferencias en el fondo genético pueden afectar al resultado. Por ello, nuestro objetivo principal fue estudiar estas tres bombas de eflujo (Tap, Stp y P55) en el microorganismo del que provienen, *M. tuberculosis*. Se construyeron, derivadas la cepa de referencia *M. tuberculosis* H37Rv, mutantes knock-out para cada una de las bombas, así como sus respectivas complementadas, además de las cepas que sobreexpresan el gen de la bomba de eflujo. Los ensayos de sensibilidad a antibióticos realizados con estas cepas confirmaron la mayoría, aunque no todos, de los resultados en M. *bovis* BCG. Además, se llevaron a cabo experimentos de infección en modelo celular y de ratón, para comprobar si la deleción del gen de la correspondiente bomba de eflujo afectaba a la virulencia de *M. tuberculosis*. Atendiendo al modelo celular, las tres bombas de eflujo parecían ser necesarias para la virulencia completa de H37Rv; sin embargo, en modelo de ratón inmunocompetente, solamente el mutante knock-out de P55 resultó ser atenuado.

En búsqueda de otra estrategia para detectar posibles sustratos de bombas de eflujo de *M. tuberculosis*, decidimos intentar expresarlas en *Escherichia coli*. Además de los ensayos de Concentración Inhibitoria Mínima, se realizaron varios ensayos que permiten visualizar la acumulación y el eflujo de compuestos el tiempo real. En caso de éxito, esta técnica permitiría comprobar rápida y fácilmente una batería de sustratos potenciales de la bomba de eflujo. Desafortunadamente, no se obtuvieron resultados significativos; es necesario continuar con varios experimentos para confirmar la expresión de las bombas de eflujo micobacterianas en *E. coli*. En paralelo, caracterizamos el fenotipo de dos mutantes knock-out para las bombas de eflujo MmpL7 y MmpL10. La cepa con el gen *mmpL7* delecionado resultó tener una aparentemente mayor velocidad de crecimiento, además de que formaba menos agregados al cultivarse en medio líquido que su respectiva cepa wild-type.

Finalmente, se realizó un análisis de cepas clínicas resistentes a estreptomicina. Se secuenciaron los tres genes relacionados con resistencia a estreptomicina (*rpsL, rrs y gidB)* en búsqueda de posibles mutaciones. Un cuarto gen se incluyó en el experimento: *Rv1258c*, que codifica la bomba de eflujo Tap, y que previamente se había descrito que r está implicado en la resistencia a estreptomicina. Nuestra hipótesis era que una posible mutación en el promotor o en el gen podía conferir resistencia a este antibiótico. Aunque no encontramos ninguna mutación que cumpliera este criterio, sí que descubrimos una mutación que estaba presente en varias cepas. El análisis en detalle reveló que este SNP (Single Nucleotide Polymorphism), denominado Tap^{580}, era específico de las cepas de la familia Beijing. Se diseño un método PCR-RFLP que permite detectar fácil y rápidamente esta mutación, y posteriormente se validó con un lote de 220 DNA de aislados clínicos pertenecientes a diferentes familias de *M. tuberculosis*.

SUMMARY

Efflux pumps are membrane proteins that transport different substrates to the outside of bacteria. It has been reported that they can have a role both in intrinsic and acquired resistance to several drugs, and can be implicated in colonization and persistence of pathogenic bacteria in the host. In adittion, other physiological processes are also affected by these membrane proteins, depending on their natural substrates.

Nowadays, the drug-resistant *Mycobacterium tuberculosis* strains have become a huge health problem. Not only Multi- and eXtensively-Drug Resistant strains, but also *M. tuberculosis* virtually resistant to all antituberculous drugs have been reported. Therefore, a global effort has to be made in order to control this emergency situation. Several strategies are in progress: the development of new anti-TB drugs and Directly Observed Treatment Short courses (DOTS) are among them. A better understanding of the mechanisms of drug-resistance would provide useful tools to improve therapy.

The MFS efflux pumps Rv1258c (Tap), Rv2333 (Stp) and Rv1410c (P55) from *M. tuberculosis* have been previously studied in the heterologous host *M. bovis* BCG, the vaccine strain. A clear role in intrinsic resistance was observed for several drugs. Although this a good approximation, since the high percentaje of identity between *M. tuberculosis* and *M. bovis* BCG, the little differences in the genetic background could affect the results. For this, our main objective was to study these three efflux pumps in their own microorganism, *M. tuberculosis*. Knock-out and complemented strains, along with overexpression strains were generated for Tap, Stp and P55, derived from the virulent *M. tuberculosis* reference strain H37Rv. Susceptibility assays confirmed most, but not all, of the results obtained in *M. bovis* BCG. Infection assays were performed in cell and mouse model, to test if the deletion of the efflux pump gene affected pathogenicity. According to cell model, the three efflux pumps seemed to be necessary for complete virulence of *M. tuberculosis*; nontheless, in immunocompetent mouse model, only P55-knock out proved to be attenuated.

In search for another strategy to detect substrates of efflux pumps from *M. tuberculosis*, we decided to try to express them in *Escherichia coli*. In addition to Minimun Inhibitory Concentration assays, a experiment in which the accumulation and efflux of molecules can be observed in real time was optimised. If successful, the technique would allow to easily and quickly test a range of potential substrates of the target efflux pump. Unfortunately, no significant results were obtained and further experiments would be required to confirm

the expression of the mycobacterial efflux pumps in *E. coli*. In parallel, two *M. tuberculosis* knock out mutants for the efflux pumps MmpL7 and MmpL10 were phenotypically characterised. Interestingly, the strain which had mmpL7 disrupted showed an apparent increase in the growth rate, as well as less aggregates than the wild type strain when it was grown in liquid medium.

Finally, an analysis of streptomycin-resistant clinical isolates was done. The genes already related to streptomycin resistance (*rpsL*, *rrs* and *gidB*) were sequenced and analysed in search of mutations. A fourth gene was included in this experiment: *Rv1258c*, encoding Tap efflux pump. Since this transporter is implicated in streptomycin resistance, our hypothesis was that a possible mutation in the promoter or in the gene could be conferring resistance. Although we didn't find any mutations in *tap* gene fulfilling this criteria, we did notice a mutation that several clinical isolates had in common. Further research revealed that this Single Nucleotide Polymorphism (SNP), called Tap^{580}, was specific of Beijing lineage of strains. A quick PCR-RFLP method to easily detect this mutation was designed, and checked with a lot of 220 DNA extractions of clinical isolates belonging to different lineages of *M. tuberculosis*.

Introduction

History of Tuberculosis

The disease is caused by *Mycobacterium tuberculosis*, a bacillus whose history is closely related to that of mankind, as it has infected humans for thousands of years. Pathological evidence of TB has been found in Egyptian mummies dating back five millennia, and a specific DNA sequence unique to *M. tuberculosis* has been identified in Egyptian and Peruvian mummies dating back to 500-1500 Before Christ (BC). By the first millennium BC, tuberculosis was endemic all around the world, and named *phtisis*, the Greek term for consumption. At that time (460 BC), *phtisis* was the most widespread disease in Ancient Greece.

It was not until XVII and XVIII centuries After Christ (AC) that there was an advance in the understanding of the disease. The 17th century German physician Franciscus Sylvius firstly described the "tubercles" as characteristic lesions in the lung of people suffering from phtisis, and almost 200 years later, this discovery will further name the disease as "tuberculosis". Some studies performed between 1600 and 1800 AC, had suggested that the main infective agent was the sputum of ill patients, and it was even proposed that tuberculosis was caused by some *animacula*, or *wonderfully minute living creatures*. This hypothesis, published in 1719 by Benjamin Marten, was considered ridiculous.

From second half of the XVIII century until the end of the XIX, TB was a prevalent disease in Europe and North America, and was called *white plague* for the extreme paleness of people suffering from this disease. But XIX century is also the century of scientific advances and a marked decrease in deaths caused by tuberculosis was about to happen.
It was in 1882 when Robert Koch first observed *M. tuberculosis* (that was also named "Koch bacillus" in his honor) under the microscope, and published a demonstration that it was the etiological agent of tuberculosis. He had previously presented the Koch's Postulates, describing how an infectious microorganism isolated from a lesion of a person or animal suffering the disease, can reinfect a healthy animal; those postulates are currently the basis of medical microbiology.

Since then, great advances in fight against TB have happened incessantly. In 1921 Albert Calmette and Camille Guerin obtained the current vaccine against TB, named BCG (Bacille de Calmette et Guerin). In 1944 Schatz and Waksman discovered streptomycin, an antibiotic with antimycobacterial activity. This fact, followed by the discovery of isoniazid in 1952 and rifampicin in 1967, marks a new period for tuberculosis. It turned into a

curable disease and new cases decreased considerably until the '80s. Appearance of HIV in addition to other factors, such as migrations, caused then the reemergence of tuberculosis and other diseases, and in 1993 it was declared an emerging global health problem by the WHO (Fig. 1.).

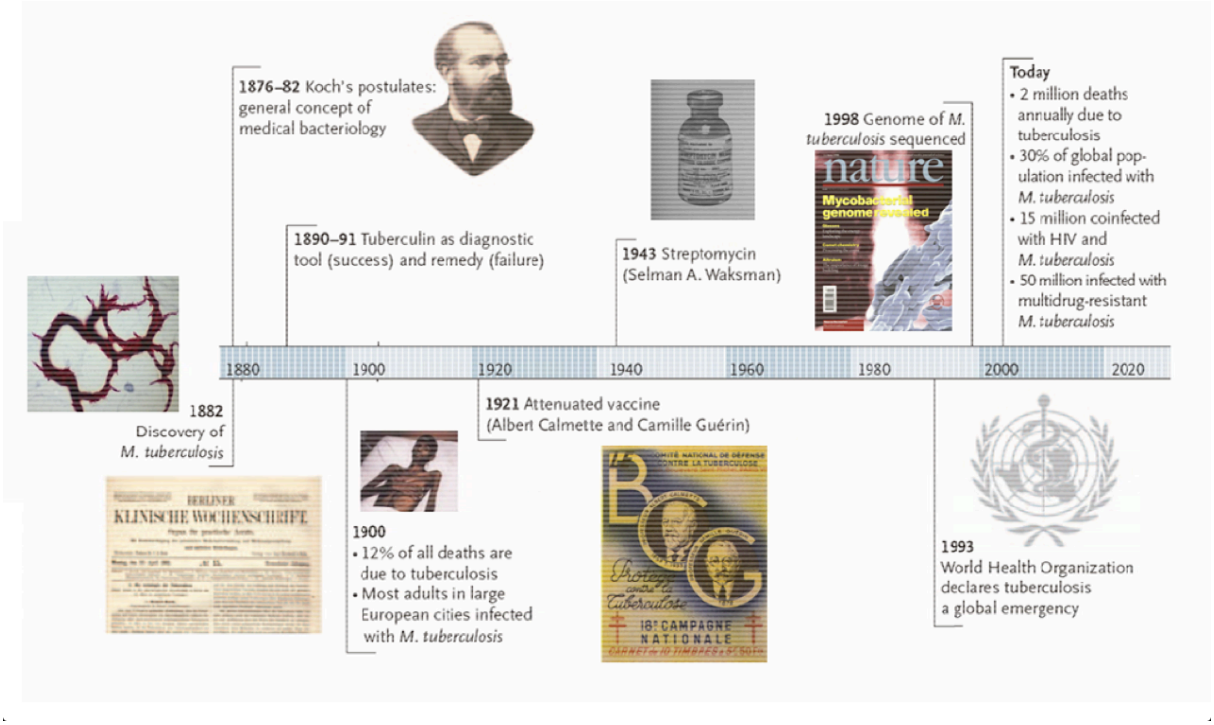

Fig. 1. Timeline in TB research. It shows the most relevant highlights in the fight against the disease. Adapted from (51).

Nowadays, the emergence of strains that are resistant to the current therapy complicates the aim of TB eradication. However, we count currently on great scientific advances, including the knowledge of the complete genome of *M. tuberculosis* (27) that will allow us to better understand the biology and molecular mechanisms of the bacillus, to efficiently win this fight.

Mycobacteria and the tubercle bacillus

Mycobacteria are acid-fast bacilli belonging to the genus *Mycobacterium*, family *Mycobacteriaceae*, included into the suborder *Corynebacteriaceae*, order *Actinomycetales* (94). Philogenetically they belong to the gram-positive group of bacteria, however, due to their highly hydrophobic cell wall, different from those of both gram positive and gram negative bacteria, they are only weakly stained by gram staining.

The genus *Mycobacterium* comprises more than 120 species (98). They include nonpathogens, e.g. *Mycobacterium smegmati*s, as well as highly successful pathogens e.g. *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium ulcerans*, the etiologic agents of tuberculosis, leprosy and Buruli ulcer, respectively. Attending to the rate of replication, mycobacterial species can be divided in two groups: slow-growing mycobacteria, with a doubling time higher than 13 hours, like *M. tuberculosis,* and rapid-growing mycobacteria, with a generation time between 2 and 5h, like *M. smegmatis*.

Colony morphology is variable among species, from rough to smooth and pigmented to non-pigmented. Mycobacteria inhabit various environmental reservoirs, like ground and tap water, soil, animals and humans. They can grow in fluid environments, usually forming clumps because of their high hydrophobic cell envelope; some mycobacteria are invasive, since they are able to multiply inside macrophages, such us *M. tuberculosis*.

Classically, the genus *Mycobacterium* has been organised in a phylogenetic tree attending the homology level of 16S RNA gene (Fig. 2.) (38); in adittion, other genes have been analysed as well in order to reach a more robust phylogeny (99).

M. tuberculosis is an aerobic bacillus, catalase and nitrate reductase positive, non-motile and non-sporulated, of 2-5μm in length and 0.2-0.5μm in width. Its doubling time (~24h) classifies it as a slow-grower, which needs 4 weeks to form a colony on solid medium. Until recently, it was believed that mycobacterial species don't sporulate, but a study by Ghosh *et al.*(39) revealed that *M. marinum* and most likely *M. bovis* BCG, actually can form spores.

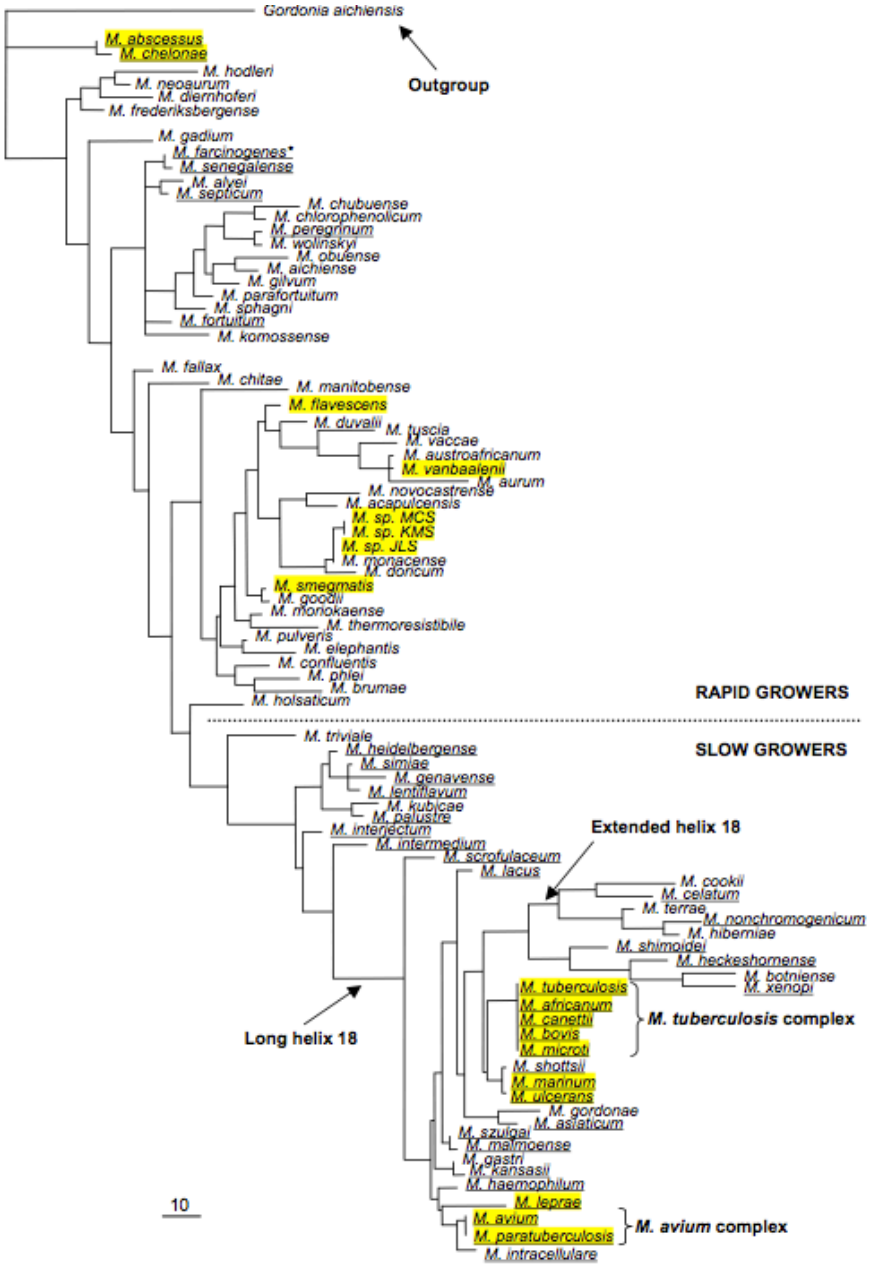

Fig 2. Phylogenetic tree of the genus *Mycobacterium* based on 16S RNA homology. Rapid and slow growers are separated with a dotted line, virulent strains are underlined. Adapted from (38).

Mycobacterial cell envelope

The basic architecture of the mycobacterial cell envelope differs from the ones of gram positive and gram negative bacteria. The *Mycobacterium tuberculosis* envelope, from the inside to the outside, consists of a plasma membrane, similar to other plasma membranes (28); a cell wall composed of three covalently linked macromolecules (peptidoglycan, arabinogalactan and mycolic acids) and noncovalently linked lipids and proteins; and a capsule consisting of polysaccharides, proteins and lipids (Fig. 3.) (72). The

lipopolysacharides lipomannan (LM) and lipoarabinomannan (LAM) are not covalently attached to the mycobacterial envelope, and their localization remains unclear (40). The non-covalent associaton of mycolic acids and complex free lipids conforms an atypical outer membrane (45). In *M. tuberculosis,* these free lipids include the phthiocerol dimycocerosates (PDIM) and the closely related phenolic glycolipids (PGL), the trehalose ester families that include sulfolipids (SL), diacyltrehaloses (DAT) and polyacyltrehaloses (PAT), and the family of mannosyl-ß-1-phosphomycoketides (46). The trehalose dimycolate (TDM) or "cord factor", named so before discovering its chemical structure, is also a free lipid implicated in cording, and constituted the first virulence factor of *M. tuberculosis* (41).

Important functions are related with the mycobacterial envelope, such us defining the shape of the cell and providing mechanical and osmotic protection. Other important function is the transport of molecules, including nutrients, ions, antibiotics and toxic metabolites. Its unually high hidrophobicity makes it an efficient barrier for antibiotics, thus contributing to intrinsic drug resistance. Regarding *M. tuberculosis*, when infecting macrophages, the cell wall is the primary interface between the bacterium and the host, constituting the first line of defense against host attacks. In adittion, some complex free lipids, such us PDIM, are known to be virulence factors, and others contribute to modulate the immune response from the host (9).

Such a lipid-rich coat explains the tendency of mycobacteria to form aggregates when grown in liquid medium, as well as the property of acid fastness. The characteristic cell wall of mycobacteria retains the dye carbol-fuchsin after a wash with an alcoholic solution in acid medium (Ziehl-Neelsen stain), whereas the rest of bacteria are quickly decolorized. It was recently suggested that this is a consequence of the density of mycolic acids (111).

In addition, the fixation of the dye neutral red is used as a marker of virulence, since this dye indicates important alterations in the cell envelope of *M. tuberculosis*. Specifically, neutral red is unable to fix to the cell envelope when bacteria are deficient in more than one type of methyl-branched lipids, which are related to virulence, such us PDIM (26).

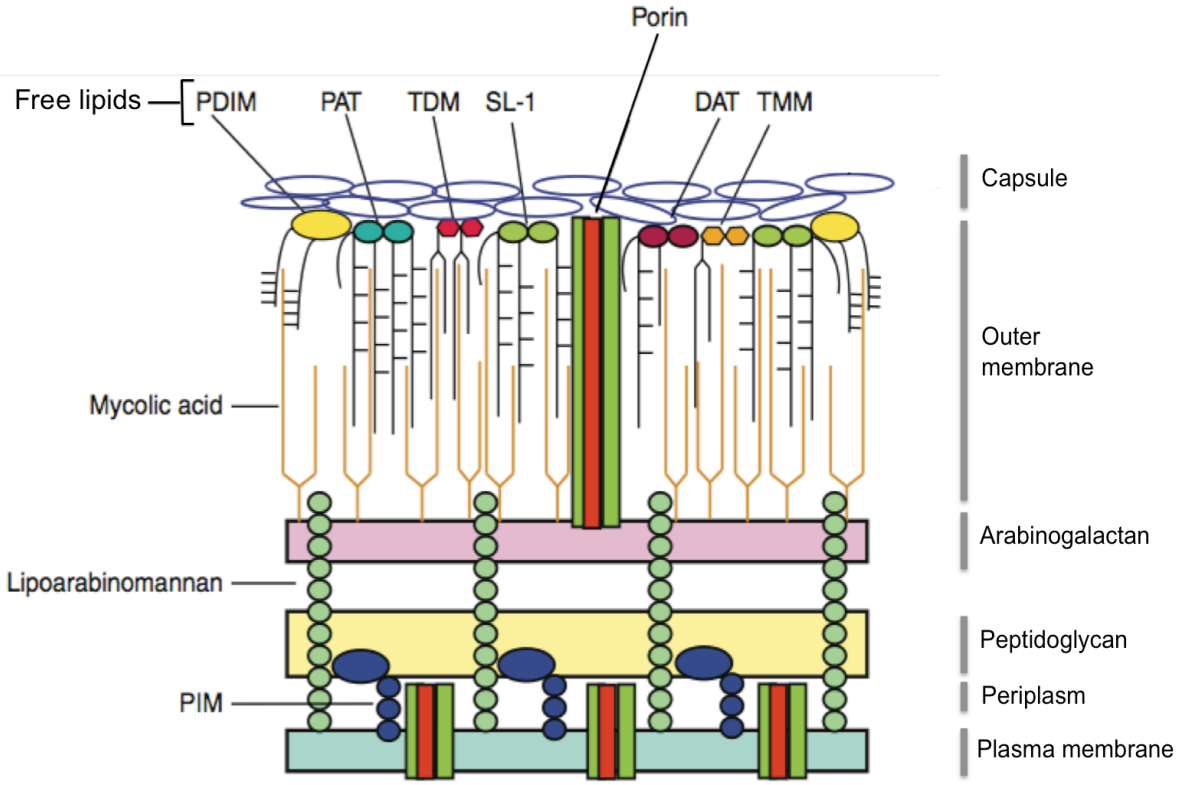

Fig. 3. Schematic representation of cell envelope of *M. tuberculosis*. Adapted from (72)

Mycobacterium tuberculosis complex and phylogeny of *M. tuberculosis*

The Mycobacterium tuberculosis complex (MTBC) is composed by all the etiologic agents of tuberculosis, and currently includes nine species.

Species	Host
M. tuberculosis	Human
M. africanum	Human
M. canettii	Human
M. bovis	Cattle
M. caprae	Goat
M. microti	Vole
M. pinipedii	Seal and sea lion
M. mungi	Mongoose
M. orygis	Oryx

Table 1. Species belonging to MTBC (5, 103)

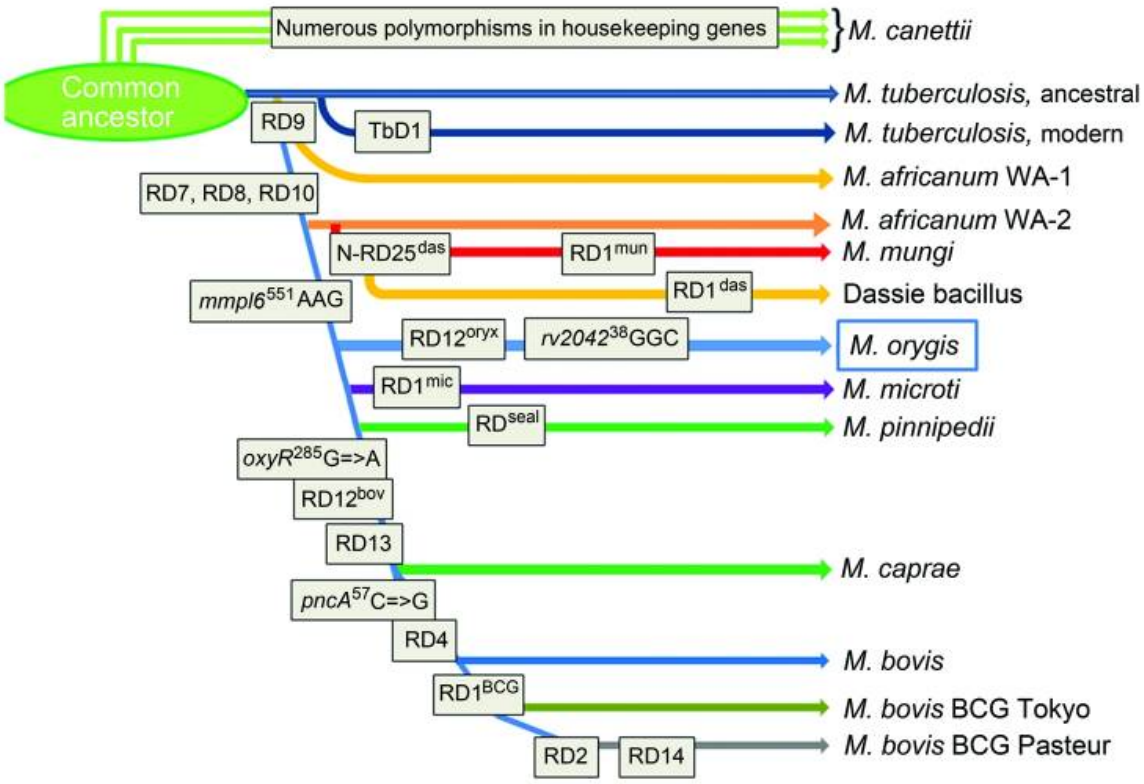

Fig 4. Updated phylogeny of the MTBC. Adapted from (103).

The development of techniques such us IS6110 RFLP (102), spoligotyping (42, 49), Mycobacterial Interspersed Repetitive Units (MIRU) (96) and more recently, Single Nucleotide Polymorphism (SNP) (36, 44) and Large Sequence Polymorphism (LSP) genotyping (66, 100), have led to the intraespecific classification of *M. tuberculosis* strains into several lineages: East African-Indian (EAI) lineage, Beijing lineage, Central-Asian (CAS) or Delhi lineage, Haarlem family, Latin American and Mediterranean (LAM) family, X family, T families and others. (83)

Infection by *M. tuberculosis*

Tuberculosis is a contagious disease (114). Infection with *M. tuberculosis* occurs through inhalation of aerosols containing the bacteria which are spread by persons with active pulmonary TB. After inhalation, bacteria are settled in the alveoli and disseminated by the lymphatic circulation. Further dissemination to other parts of the lung and occasionally to other organs is achieved by haematogenous circulation. The most common form of the disease is pulmonary tuberculosis, although TB-meningitis, miliary (disseminated) tuberculosis, lymphadenitis, osteomyelitis and Pott's disease (affected bones) also occur (114). Primary infection leads to active disease in about 10% of infected individuals, in 80%

of the cases in the period of two years (126). In the remaining 90%, the immune system controls the infection, and the individual is non- infectious and asymptomatic. In this clinical state the TB bacilli can lie dormant for years (latent TB) (103). However, when the immune system is weakened, the latent infection can reactivate (114, 126). In an HIV-infected person the risk of reactivation of latent TB is higher than 10% per year, compared to a lifetime risk of 10-20% for HIV- negative individuals (24, 112, 126).

Once the bacteria are phagocytosed by alveolar macrophages, mediated by specific macrophage receptors (37, 39, 63, 111), a localized proinflammatory response that leads to recruitment of mononuclear cells from neighboring blood vessels is induced. These cells will build the granuloma (necessary to contain infection) which consists of a group of infected macrophages, surrounded by foamy giant cells (multinuclear macrophages loaded with lipids) and macrophages with a layer of lymphocytes delineating its periphery (102-104) (Figure 2).

Activated macrophages promote phagosomal maturation by a process that finally involves fusion with lysosomes and formation of the phagolysosome. These vesicles provide a hostile environment for the bacilli including acid pH, reactive oxygen intermediates (ROI), lysosomal enzymes and toxic peptides. *M. tuberculosis* has evolved to survive within macrophages by arresting the normal phagosomal maturation at an early stage, thereby restricting its acidification and limiting fusion with lysosomes (91, 99). Modulation of phagosome maturation seems to be mainly mediated by both mycobacterial cell-wall lipids and other bacterial effectors (e.g., SapM or PknG) (47, 133, 134). Additionally, the screening of shotgun libraries has enabled the isolation of several *M. tuberculosis* mutants unable to induce phagosomal arrest (97, 119). Apart from surviving within the macrophage, pathogenic mycobacteria have also been suggested to escape into the cytosol in an ESX-1-dependent manner (129). In addition, *M. tuberculosis* reduces phagosome acidification by the exclusion of the proton- ATPase (120) and decreases ROI and nitric oxide (NO) synthesis and cytokine production by modulating Ca^{2+} fluxes in infected macrophages (77). Moreover, *M. tuberculosis* has evolved multiple strategies to detoxify ROI and RNI (38). Recently, authophagy (30, 58) and apoptosis (69) have been postulated as effective antimycobacterial mechanisms.

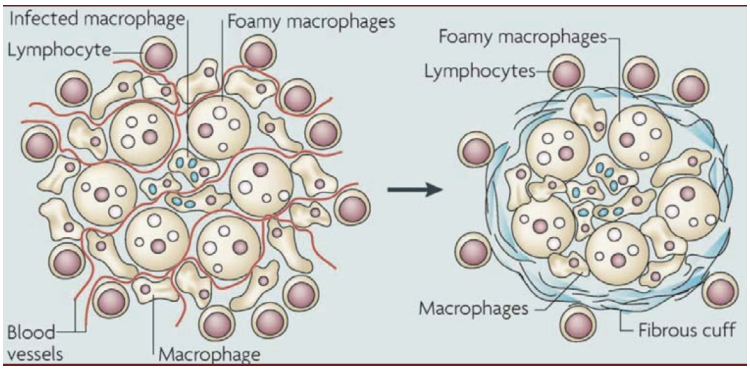

Fig. 5: Progression of TB granuloma. In the early stage, the granuloma has a core of infected macrophages enclosed by foamy macrophages and other mononuclear phagocytes, surrounded by lymphocytes. As the granuloma matures, it develops a fibrous capsule that encases the macrophage core and excludes the majority of lymphocytes from the center of the structure. Adapted from (85).

Genomics of *M. tuberculosis*

The complete genome sequence of the most studied and the best understood strain of *M. tuberculosis,* H37Rv, was obtained in 1998 (114) and revised and re-annotated in 2002 and it is in continuous revision (as of August 2010; NCBI Entrez Genome Project). Complete genome sequences are now available for five *M. tuberculosis* strains, CDC1551, F11, H37Ra, H37Rv and KZN1435, and sequencing of 71 other *M. tuberculosis* genomes is in progress providing a valuable tool for research using genetic techniques (71). This information is available in sequencing websites, as well as in several online databases (http://tuberculist.epfl.ch/, http://www.ncbi.nlm.nih.gov/ or http://www.microbesonline.org/).

The genome comprises 4,411,532 bp, with a G+C content of 65.9% (22). Only a few regions show a skew in this G+C content. A conspicuous group of genes with a very high G+C content (> 80%) appear to be unique in mycobacteria and belong to the family of PE or PPE proteins. In turn, the few genes with particularly low (< 50%) G+C content are those coding for transmembrane proteins or polyketide synthases. This deviation to low G+C content is believed to be a consequence of the required hydrophobic amino acids, essential in any transmembrane domain, that are coded by low G+C content codons. The last re-annotation identified 4090 genes thought to encode 3,989 proteins and 71 stable RNAs (Figure 4) (71). Some outstanding features include the great abundance of genes involved in lipid metabolism which is indicative of the highly active metabolism mainly in relation with the cell envelope. There are genes encoding for 250 distinct enzymes involved in fatty acid metabolism, compared to only 50 in the genome of *Escherichia coli* (21). The PE- PPE family of proteins, which represents approximately 4% of the genome, includes 168 genes. The names come from Pro-Glu (PE) and Pro-Pro-Glu (PPE) sequences found in the two conserved N-terminal regions in each of these protein families that are approximately 110

and 180 amino acids long, respectively. Although the function of the members of the PE and PPE protein families has not been established, they are suggested to be involved in antigenic variation and disease pathogenesis (29). Tubercle bacilli also contain complex regulatory machinery comprising 11 two component systems, 11 serine/threonine protein kinases, 13 σ factors, and over 100 repressors and activators, suggesting a tight regulation of transcription in response to different stimuli. Interestingly, the analysis of the DNA metabolic system of *M. tuberculosis* indicates a very efficient DNA repair system, in other words, replication machinery of exceptionally high fidelity. The genome of *M. tuberculosis* lacks the MutS- based mismatch repair system. However, this absence is overcome by the presence of nearly 45 genes related to DNA repair mechanisms (88), including three copies of the *mutT* gene. This gene encodes the enzyme in charge of removing oxidized guanines whose incorporation during replication causes base-pair mismatching (21, 88). Repetitive DNA is very important in mycobacterial genomes. Elements like Tandem Repeats, Interspersed Repeats, and mobile genetic elements are common. In H37Rv, 56 loci with similarity to IS elements have been found that can be classified into major IS families (IS3, IS5, IS605 or IS21). Mobile genetic elements will be widely analyzed in the following section as they could have an important role in genome plasticity and the virulence of the bacilli.

Fighting against tuberculosis

At the middle of the twentieth century the eradication of tuberculosis was considered feasible. Although several problems were anticipated, it was thought possible to strengthen the control programs of the disease to the point of its total elimination. The failure of these predictions became obvious when in 1986 the downward trend of the incidence rate of tuberculosis reversed. The reasons are several and complex, including the arrival and spread of HIV infection, the immigration of people from high-prevalence countries and the deterioration of tuberculosis control. Nowadays, the goal to eliminate tuberculosis has been set for 2050, but it will not be met with present strategies and instruments, thus a strong effort is being made to improve both prevention and treatment of this deasease.

Vaccination

BCG is one of the most widely used vaccine today (> 80 % of neonates and infants in some countries). Although it does not prevent primary infection or reactivation of latent pulmonary TB, the principal source of bacillary spread in the community, BCG protects

against disseminated forms of TB, especially against meningitis in children. For that reason, BCG vaccination is still recommended by the WHO and applied widely (116, 124).

After its first use in humans in 1921, propagation of BCG in non-standardized conditions in different laboratories all around the world gave rise to different variant strains that differ in genotype and phenotypic characteristics (9). Strain differences could be one cause of the variable protective efficacy afforded by BCG (45), but other factors such as population genetics or exposure to environmental mycobacteria could be implicated (29).

Due to this inconsistency in protective efficacy, and to the inability of BCG to prevent pulmonary forms of tuberculosis and consequently, to prevent transmission of the disease, great efforts are being made by governments, research institutions and private foundations for constructing and testing new promising vaccine candidates.

Treatment

The aims of treatment of tuberculosis are:

- to cure the patient and restore quality of life and productivity.
- to prevent death from active TB or its late effects.
- to prevent relapse of TB.
- to reduce transmission of TB to others.
- to prevent the development and transmission of drug resistance.

The antibiotics active against tuberculosis can be divided in first-line and second-line drugs. First line agents are generally those with the greatest bactericidal activity and they form the core of TB treatment. Second-line agents are normally bacteriostatic, they have more adverse effects, and are commonly used in case of disease caused by strains resistant to first-line drugs. (Table 2.)

First-line drugs	Second-line drugs
Isoniazid	Ethionamide
Rifampin	Cycloserine
Pyrazinamide	Para-amino salicylic acid
Ethambutol	Capreomycin
Streptomycin	Kanamycin
	Amikacin
	Quinolones (Moxifloxacin, Levofloxacin, Gatifloxacin, Ciprofloxacin)
	Linezolid

Table 2. Drugs used in treatment of tuberculosis. (50)

Tuberculosis treatment requires long courses (around 6 to 12 months) of multiple antibiotics. It consist of an initial intensive phase of treatment (2 months) with RIF, INH, PAZ and either EMB or SM designed to kill actively growing and semi-dormant bacilli, followed by a continuation phase (4 months) with RIF and INH to eliminate residual bacilli and reduce the number of relapses. To complete the treatment it is critical to reduce the development of acquired drug resistance and therefore trained individual personnel supervises that the patient takes each dose of medication. This strategy is known as directly observed treatment short-course (DOTS) and was promoted as the official policy of the WHO in 1991 (137).

Drug resistance and tolerance of *M. tuberculosis*

Intrinsic resistance

The thick and highly hydrophobic mycobacterial cell wall envelope is an effective permeability barrier to many antibiotics, being one of the main causes of the high, intrinsic antibiotic resistance in mycobacteria (20). But there are other mechanisms or genes likely contributing to it.

M. tuberculosis is known to contain numerous genes encoding efflux pumps belonging to different families (30, 36). It has been reported that overexpression of some genes encoding efflux pumps confers resistance to different drugs in different mycobacteria, as shown in Table 2, and it is becoming evident the implication of efflux mechanisms in the intrinsic resistance to drugs.

In addition to efflux pumps, mycobacteria produce some drug-modifying enzymes, such as ß-lactamases, responsible for the intrinsic resistance to ß-lactams drugs in *M. tuberculosis* (73).

The gene *erm37* (or *ermMT*) encodes a 23S rRNA methyltransferase, which is responsible for intrinsic resistance to macrolides (9, 23) not only in *M. tuberculosis* but also in other mycobacteria (97-99).

Aminoglycoside modifying enzymes are also present in the chromosome of *M. tuberculosis* and other mycobacteria but their role in resistance is unclear, as two of those genes, *aac(2')-Ic* from *M. tuberculosis* and *aac(2')-Id* from *M. smegmatis*, were studied in M. smegmatis and only *aac(2')-Id* was correlated with aminoglycoside resistance (6, 90).

It has been reported that *whiB7,* a transcriptional activator, was responsible for intrinsic resistance to several antibiotics of different structures, like erythromycin, chloramphenicol, clarithromycin, spectinomycin, streptomycin or tetracycline (94). It has been shown that *whiB7* is a regulator that may be involved in the induction of some previously described genes, including the gene encoding for the efflux pump tap, or the gene *erm37*. There are 7 *whiB* genes in M. tuberculosis (whiB1-whiB7) induced by different stress conditions and antibiotics (52, 134), and some homologous ones which have already been found in *M. smegmatis* (114).

Acquired resistance

Bacteria can become resistant to antibiotics by several strategies: i) target modification, ii) target overexpression, iii) barrier mechanisms (reduce uptake or increased efflux), iv) drug-inactivating enzymes, v) inactivation of drug-activating enzymes. Genes conferring resistance can be located in plasmids or transposons, and therefore can be transferred horizontally from one strain to another (50) .

M. tuberculosis is able to acquire resistance by mutation in its chromosomal genes; this will be further detailed in the mechanism of resistance of each antituberculous antibiotic. No horizontal transfer of resistance genes had been reported for *M. tuberculosis* (50).

Tolerance

Tolerance is defined as the phenotypic resistance or non-susceptibility to a drug mediated by changes in a cells's physiological state, which is often associated with non-replicating state as in persisters (50). It has been reported that, upon macrophage infection, bacterial efflux pumps confer tolerance (Fig. 6) (1); however, the mechanisms underlying this transient resistance are not fully understood.

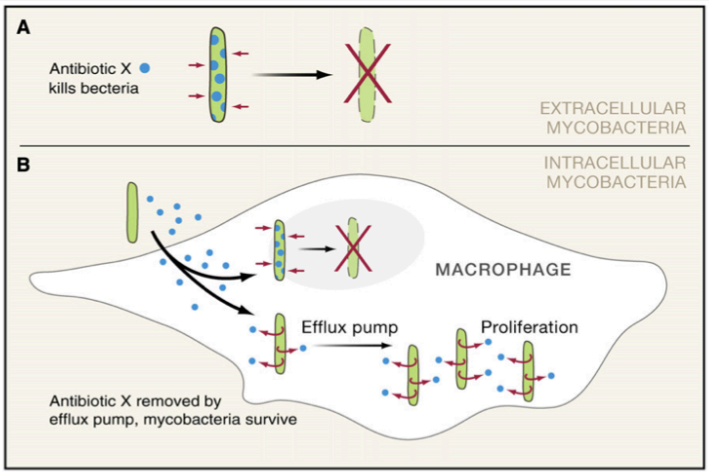

Fig. 6. Antibiotic Tolerance in Replicating Mycobacteria Mediated by Macrophage-Induced Drug Efflux (A) Extracellular mycobacteria are killed and eliminated by antibiotics. (B) Mycobacteria that enter macrophages may retain susceptibility to drugs and be killed, or they may induce expression of efflux pumps, which makes them drug tolerant and able to replicate and persist in the face of otherwise lethal concentrations of antibiotics. (1, 76)

Antibiotics against tuberculosis

In this section, mechanism of action and drug resistance are explained for each anti tuberculosis drug.

First-line antibiotics

Isoniazid

Isoniazid (isonicotinic acid hydrazide, INH) is one of the most-widely used first-line TB antibiotics, utilized both in standard chemotherapy as well as in chemoprophylaxis (166). It is highly active against growing tubercle bacilli but has little activity against resting bacilli in stationary phase or under anaerobic conditions (93).

Isoniazid is a prodrug that becomes active thanks to the action of the catalase peroxidase encoded by the gene *katG* (167). The current mode of action proposes that, when activated, INH binds to NAD forming an INH-NAD adduct. This adduct binds and inhibits InhA (an enoyl-acyl carrier protein reductase involved in mycolic acid biosynthesis),

consequently inhibiting mycolic acid biosynthesis and inducing cell death (154) (Fig. 4). InhA is accepted to be the target of INH.

Loss of KatG activity implies a failure in activating INH (155), hence mutations in the katG gene are cause of resistance. Indeed, between 20 and 86% of INH resistant strains (depending on the geographical region) contain a mutation in the katG gene, being the mutation S315T the most common.

Fifteen to forty-six percent of the resistant mutants present a mutation on the promoter region of the inhA gene. Those mutations have led to an increased expression of this gene, and cause low level of INH resistance (170) and co-resistance with ethionamide, as both drugs act on the same target InhA (154).

Although most INH resistant strains have mutations in *katG* and *inhA*, other genes have been postulated to have some implication in INH resistance, like *ndh-II, ahpC* or *kasA*. Some efflux pumps, including *mmpL7* or the pump component *iniA* have been reported to be involved in INH resistance (67).

Rifampicin

Rifampicin (RIF) is a semisynthetic first- line antibiotic for the treatment of TB but is also a broad spectrum antibiotic active against a wide range of bacteria.

RIF is active against growing *M. tuberculosis* and stationary phase bacilli with low metabolic activity. RIF inhibits RNA synthesis by binding to the bacterial RNA polymerase (25).
Resistance to RIF in M. tuberculosis, as in many other bacteria, is caused by mutations in the rpoB gene that encodes the ß-subunit of the RNA polymerase. Those mutations are found in about 96 % of RIF-resistant TB isolates (146).

In addition, it has been found that the deletion of the Rv1410c gene encoding the P55 efflux pump made *M. bovis* BCG more susceptible to a range of toxic compounds including rifampicin (117).

Ethambutol

Ethambutol (EMB) is a mycobacteria-specific drug with no apparent activity against other bacteria. It has a bacteriostatic activity only against actively growing bacilli, having no effect on non-replicating bacilli (164).
The proposed target of EMB is the arabinosyl transferase, an enzyme involved in synthesis of arabinogalactan - the major polysaccharide of the mycobacterial cell wall (91). EMB then inhibits the synthesis of arabinogalactan, interfering with the polymerization of cell wall arabinan (144).

The enzyme arabinosyl transferase is encoded by the gene embB, which is part of an operon containing the genes *embC-embA-embB* in *M. tuberculosis*. Up to 68% of the EMB-resistant clinical isolates presents mutations in the embCAB operon (147,140). Other genes including *Rv0340, rmlD* or *rmlA2,* have been found to be associated with EMB resistance (115), though they are not significant.

Around 25% of EMB-resistant *M. tuberculosis* strains do not have mutations in any of the genes described, suggesting that there may be other mechanisms involved in EMB resistance.

Pyrazinamid

Pyrazinamide (PZA) is a first-line antibiotic used in the initial phase of treatment that is very active against *M. tuberculosis*, while other bacteria, including other mycobacteria, are not susceptible. PZA is only active at acid pH (86), showing activity *in vivo* but not *in vitro*. Uncommonly, it kills non-growing bacilli with low metabolic activity more efficiently than actively growing bacilli (171).

The proposed mode of action of PZA (Fig. 6) suggests that it enters the bacilli and is converted into pyrazinoic acid (POA) by the action of pyrazinamidase/nicotinamidase enzyme, encoded by the *pncA* gene. POA gets out of the cell by passive diffusion and weak efflux (in *M. tuberculosis* it seems to be rather defective), and in an acidic extracellular environment, a portion of POA becomes protonated to HPOA, the uncharged form that permeates through the membrane inside the cell. HPOA brings protons into the cell, what could cause cytoplasmatic acidification and de-energization of the membrane by collapse of the proton motive force further affecting membrane transport (168).

Thus most PZA-resistant *M. tuberculosis* strains (72 – 97 %) present mutations in the pncA gene, losing the pyrazinamidase/nicotinamidase activity and failing in the activation of the prodrug (131, 132). However, while *M. tuberculosis* is uniquely susceptible to this drug, other mycobacteria are intrinsically resistant. In the case of *M. bovis*, this resistance is due to a SNP in the *pncA* gene with respect to *M. tuberculosis*, which causes lack in PZase activity (130). In *M. smegmatis* and other mycobacteria, the resistance is suggested to be due to an active efflux of POA (169). There are still some resistant TB strains whose mechanism of resistance to PZA still has to be determined.

Streptomycin

Streptomycin (SM) is an aminoglycoside antibiotic discovered in 1944 (1) that was the first effective TB drug. Aminoglycosides are broad-spectrum antibiotics with a bactericidal activity against a variety of bacterial species including *M. tuberculosis*. SM kills actively growing bacilli, while it has no effect on non-growing or intracellular tubercle bacilli (92). Due to the severe adverse effects that can cause and also because it is an injectable drug, it has been relegated as a second-line TB drug.

SM inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome, specifically to the ribosomal protein S12 and the 16S rRNA (51). It can also cause damage to the cell membrane.

SM resistance in *M. tuberculosis* is due to mutations in the genes *rpsL* and *rrs* which encode respectively the S12 protein and the 16S rRNA of the ribosome, that is the SM target (47). Approximately 70% of the SM-resistant clinical isolates have a mutation in one of those two genes, resulting in intermediate or high level of resistance. There are still 20-30% of the resistant strains presenting low level resistance with no mutation found (164), which suggests that there are other mechanisms of resistance.

For example, Tap efflux pump has been shown to confer low level resistance to SM when overexpressed (3). In addition, it has recently been found that mutations within the gene gidB (encoding a 7-methylguanosine (m(7)G) methyltransferase specific for the 16S rRNA) confer low-level streptomycin resistance by loss of a conserved m(7)G modification in 16S rRNA (102).

Despite the existence of aminoglycoside modifying enzymes, their role in clinically relevant resistance still remains unclear since none appears to have SM as a substrate.

Second-line antibiotics

Fluoroquinolones

Fluoroquinolones are drugs with a broad-spectrum bactericidal activity against a wide range of bacteria, including mycobacteria. They are known to inhibit DNA gyrase complex, as well as DNA topoisomerase IV. Mutations in gyrA and gyrB (genes encoding the two subunits of DNA gyrase) cause resistance to quinolones in M. tuberculosis, which does not have topoisomerase IV. However, there are some mutants in which DNA gyrase is not involved, and the mechanism of their resistance is not clear (41).

A new mechanism of quinolone resistance was recently identified. MfpA is a protein that binds to DNA gyrase and inhibits its activity, also giving FQ resistance (59).

Aminoglycosides

Other aminoglycosides besides SM, such as kanamycin, gentamicin, amikacin, or capreomycin – which is a peptide antibiotic but commonly grouped with the aminoglycosides - that are not first-line drugs for TB treatment, play an important role in the treatment of MDR strains or infections with mycobacteria other than *M. tuberculosis*.

Since they are aminoglycosides, and they bind to ribosomal structures at the 16S rRNA, mutations in *rrs* gene are also cause of resistance (7, 66) even though they do not cause cross resistance with SM.

Recently, it has been described that *tlyA*, a gene encoding an rRNA 2'-O-methyltransferase, is involved in capreomycin and viomycin resistance (84).

In mycobacteria there are some chromosomally encoded aminoglycoside modifying enzymes that only confer resistance to some agents in the case of *M. smegmatis* (5, 6, 90).

Cycloserine

Cycloserine is a bacteriostatic antibiotic used as a second-line agent often in combination with other second-line drugs to treat MDR TB. It inhibits the synthesis of peptidoglycan by

blocking the action of D-alanine racemase and D-alanine:alanine synthase but the genetic bases of CS resistance are unclear (164)

PAS

PAS (Para-Amino Salicilic acid) is a bacteriostatic antibiotic active against TB and other mycobacteria but not against non-mycobacterial species. It is used as a second-line drug in combination with others to treat MDR-TB.

The mechanism of action of PAS is not well understood, though two possible mechanisms have been proposed: interference with folic acid biosynthesis (120) and inhibition of iron uptake (118).

The gene *thyA,* encoding a thymidilate synthase has been reported recently to be involved in PAS resistance in some clinical isolates (120).

Ethionamide

Ethionamide (ETH) is a derivative of isonicotinic acid, and is a second-line anti TB drug. It shares a common target with INH, the enoyl-acyl carrier protein reductase (InhA) involved in mycolic acid biosynthesis. Therefore it inhibits mycolic acid biosynthesis and causes cell death.
ETH is a prodrug that needs to be activated. This activation is done by oxidation, thanks to an enzyme codified by the gene *etaA*, also called *ethA*. Consequently, mutations in *ethA* cause resistance to ETH (164).

Mutations on the promoter region of the inhA gene leading to increased expression, cause low level of ETH resistance and co-resistance with INH, as both drugs act on the same target InhA (154).

The mechanism of action and target are the same for ETH and INH, so the resistance mechanisms described for INH are valid for ETH and if common, they cause co- resistance. The different step is the activation of the prodrug, carried out by KatG in case of INH, and EthA in case of ETH.

The Global Problem of TB Drug Resistance and the Need of New Antibiotics

MDR-, XDR- and TDR-TB strains

Multi Drug-Resistant Tuberculosis (MDR-TB), is the disease caused by bacilli resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs.

It can take up to two years to treat with drugs that are more toxic, more expensive, and less effective than the first-line ones. If the drugs to treat MDR-TB are mismanaged, further resistance can occur. In 2006 a new type of resistant TB was described - XDR-TB (eXtensively Drug-Resistant TB) – which is more dangerous, and threatens all the TB control efforts (156). XDR is defined as: "resistant to at least rifampicin and isoniazid, in addition to any fluoroquinolone, and at least one of the three following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin" (157).

Unfortunately, XDR-TB strains with adittional second-line drug resistance had also been found. Some of these strains are resistant to all the tested antituberculosis antibiotics (63, 101, 107). New terms like "extremely drug resistant" ("XXDR-TB"), "super XDR-TB" and "totally drug-resistant TB" ("TDR-TB") were used to define such resistance patterns

New drugs for tuberculosis

Tuberculosis mismanagement has led to the increasing prevalence of multidrug-resistant and even extensively resistant forms of TB. There is now a worldwide recognition that new drugs for TB are urgently required, especially with the aims of shortening the treatment, and treating MDR-TB and latent infections. A great effort is being done in this direction by academic laboratories, non-profit organizations and pharmaceutical companies.

Thanks to research in drug development, a few novel compounds are being generated. WHO data show several compounds in early and late clinical trials, including TB Oxazolidinone PNU-100480 (Pfizer) and TBK-613-Quinolone (TB Alliance) in advanced preclinical trials; Diamine SQ-109 (Sequella Inc.), Linezolid (CDC TBTC, Pfizer, various Universities), Pyrrole LL-3858 (Lupin Pharmaceutical Inc.), Diarylquinoline TMC207 (Tibotec), Nitro-dihydro-imidazooxazole OPC-67683 (Otsuka Pharmaceutical Co.), Nitroimidazole-oxazine PA-824 (TB Alliance), Rifapentine (CDC TBTC, sanofi-aventis) in clinical phase I and II; and Gatifloxacin (OFLOTUB Consortium, European Commission, Lupin, WHO TDR) and Moxifloxacin (TB Alliance, Bayer, CDC TBTC, Johns Hopkins Univ, BMRC, UCL) in clinical phase III (158).

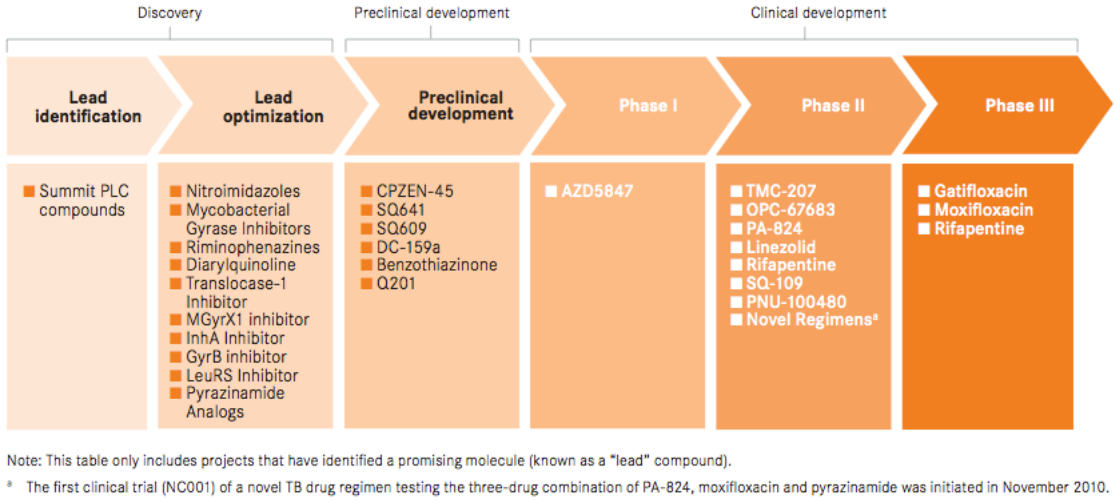

Fig 7. The development pipeline for new drugs (2011). (110)

However, there is still a need to increase the number of drug candidates. A perservering research activity in search for new targets and new compounds is being carried out. New drugs should target new pathways or enzymes to avoid cross- resistance to existing drugs. The targets have to be "druggable" proteins, with ability of inhibition by a small drug-like molecule. It is also important that the crystal structure is known or at least that it is homologue to other known proteins. In addition, the biochemical activity and biological function of the protein has to be understood, as well as what is the metabolic pathway that is going to be interfered.

Transport across the cell envelope of Bacteria

Non-polar compounds can diffuse through phospholipid bilayers, being the permeability of a particular membrane directly related with its fluidity.

Regarding mycobacteria, differential scanning calorimetry showed that the lipids in mycobacterial cell walls have very high phase transition temperatures, in the range of 60-70ºC, thus indicating the extremely low fluidity of the lipid bilayer, mainly determined by mycolic acids (59). In consequence, due to the high impermeability of the lipid bilayer of the mycobacterial membranes even for hydrophobic molecules, it is necessary the presence of channels and transporters that make possible the uptake of nutrients and efflux of toxic compounds to the outside of bacteria.

Passive transport: Porins

These proteins are located in the outer membrane of Gram negative bacteria and the outer layer of mycobacteria (35). They are water-filled pores through which hydrophilic molecules can enter the bacteria down the concentration gradient. The first porin was discovered in *E. coli*. In mycobacteria, *M. smegmatis* is known to have several porins, from which MspA represents a 70% of all pores (29). *M. tuberculosis* has also a pore-forming protein, OmpATb; however, its role as a porin is unclear (68).

Porins consist of transmembrane antiparallel ß-strands with alternating hydrophobic amino acids (facing outwards) and hydrophilic amino acids (facing inwards) assembled into ß-barrels. Connecting these ß-strands there are short periplasmic turns and longer extracellular loops. The electrostatic field resulting from hydrophilic amino acids in the ß-strands and some extracellular loops, along with the size of the pore, will determine the selectivity of the transported molecules (35).

These channels are known to have diverse functions, such as acting as receptors for bacteriocins, bacteriophages and elements of the immune system, but the main function of porins is to allow the diffusion of hydrophilic molecules into the cell. Many antibiotic molecules have low rates of diffusion through porins due to the fact that their sizes are similar to the exclusion limit; thus, limiting the uptake of antibiotics, porins contribute to intrinsic resistance (35).

Active transport: Transporters

Transporters are integral membrane proteins that transport molecules across the lipid bilayer against the electrochemical gradient. On the basis of bioenergetic and structural criteria, transporters can be classified in different groups. Attending to the source of energy, these proteins are classified in primary or secondary transporters. Primary transporters utilize ATP-hydrolysis whereas secondary transporters use transmembrane electrochemical gradient of protons or sodium ions (79).

Depending on the similarities in primary and secondary structure, transporters can be divided into five families, detailed in the following section.

Families of Transporters

ATP-Binding Cassette Superfamily (ABC)

ABC proteins are primary transporters implicated both in uptake and efflux. Its an ancient family, present in all living organisms. Their substrate profile includes sugars, amino acids, ions, drugs, polysaccharides and proteins.

These systems can be encoded by one or more genes, and they are formed by two transmembrane binding domains and two nucleotide binding domains. The TMD parts of the transporter form the transport channel and consist of a variable number (from 8 to 20) of membrane-spanning alpha-helices. The nucleotide binding domains are highly conserved among the ABC family; they bind and hydrolyse ATP, powering transport (67).

Several ABC transporters have been described to be involved in antibiotic resistance, such as MacAB-TolC in *E.coli*, or Rv2686c-Rv2688c in *M. tuberculosis* (35).

Major Facilitator Superfamily (MFS)

This ancient family of transporters can be found from bacteria to higher eukaryotes. The major facilitator superfamily (MFS) is the largest known superfamily of secondary carriers; 74 families have been described (84). MFS proteins can be divided depending on their topology in two major clusters: 12 TMS or 14 TMS (79). At the level of the superfamily, individual MFS members share low sequence similarity, having only by a pair of conserved signature sequences, whereas homology is higher among members of the same subfamily. Nontheless, it has been reported that, despite having low identity in primary sequences, all the cristalysed MFS proteins have almost the same 3D structure with two domains forming the substrate-translocation pore (56).

They are involved in the symport, antiport, or uniport of different substrates, such as monosaccharides, oligosaccharides, amino acids, peptides, phosphate esters, organic and inorganic ions and antibiotics (84). NorA of *Staphylococcus aureus* and MdfA from *E. coli* are some examples of this family of transporters (58).

All MFS transporters involved in antibiotic efflux are drug/proton antiporters (35) and they are thought to function as monomers (58). In Gram-negative bacteria, some MFS efflux systems can function as components of tripartite systems with MFPs and OM channels (e.g., EmrAB-TolC and EmrKY-TolC of *E. coli),* which can extrude substrates directly to the outside of bacteria (58). Other MFS proteins are single-component, so that they export drugs only

into periplasm. These drugs can be further taken up by the tripartite RND pumps, which will transport them accross the outer membrane (58, 97) .

Resistance-Nodulation-Cell Division Superfamily (RND)

One of the most relevant families of transporters in the clinical context is the RND family, which has been characterized best in Gram-negative bacteria (69). These pumps consist of three elements: an inner membrane pump protein with 12 transmembrane regions and two large periplasmic loops, a so-called membrane fusion protein, and an outer membrane protein that forms a channel-tunnel. The pump protein is usually trimeric and appears to work in a rotatory fashion in which individual subunits become alternately protonated and then engage and subsequently disengage substrate molecules, possibly capturing them from the cytoplasmic membrane-periplasm interface. The substrates of RND pumps are very diverse and comprise antibiotics, biocides, toxic fatty acids, bile salts, aromatic hydrocarbons, inhibitors of fatty acid biosynthesis, detergents, homoserine lactones, and dyes. The function of the so-called membrane fusion protein is not well understood but is thought to operate as a grappling hook to bring the base of the outer membrane channel-tunnel into alignment with the inner membrane pump. The outer membrane channel comprises a trimeric arrangement of a 12-stranded ß-barrel with very long coiled-coil alpha-helical segments on the periplasmic side which at their base contact the pump and are thought to open and close at the base through an iris diaphragm-like uncoiling.

Almost all RND systems are able to pump out multiple antibiotics and couple this drug efflux with proton antiport. The two best-characterized RND pumps are AcrAB-TolC of *E. coli* and MexAB-OprM of *P. aeruginosa*. However, there are numerous examples of RND efflux pumps with a demonstrated role in antibiotic resistance (35).

Small Multidrug Resistance family (SMR)

Members of the SMR family are integral inner membrane proteins from 100 to 140 amino acids in length. Each one of these proteins contains 4 TMD, and they form oligomers (dimers, trimers and tetramers) to constitute the functional transporter (10). EmrE of *E. coli* is known to function as a dimer, however, the orientation of each protomer remains unclear (58).

The SMR family can be divided into three subclasses: the small multidrug pumps, the paired SMR proteins, and suppressors of *groEL* mutant proteins. At a family level, SMR proteins have conserved sequences both in TMD1 and TMD4. The remaining TMDs also have consensus motifs, but they are related to a subgroup rather than the whole family. (10, 58)

They are proton driven, and transport quaternary ammonium compounds, commonly used disifectants and detergents and some antibiotics, such us aminoglycosides (10, 58). As MFS transporters, in Gram-negative bacteria, compounds can be transported by the SMR protein to the periplasm, and further taken by a RND efflux pump which will extrude them to the outside of the cell (97).

Multidrug and Toxic Compound Extrusion (MATE)

Transporters of MATE family have similar topology with MFS proteins, with 12 TMD. However, they constitute a different group due to the low homology at the amino acid sequence level (35). These proteins function as antiporters, most of them powered by Na^+ gradient, although some of the use the proton gradient (55), and their substrates include fluoroquinolones, aminoglycosides, and cationic dyes (35). The MATE efflux transporters are believed to be universally present in all living organisms (55). The first member of this family was NorM of *Vibrio parahaemolyticus* and other examples are YdhE from *E. coli* or PmpM from *Pseudomonas aeruginosa* (55).

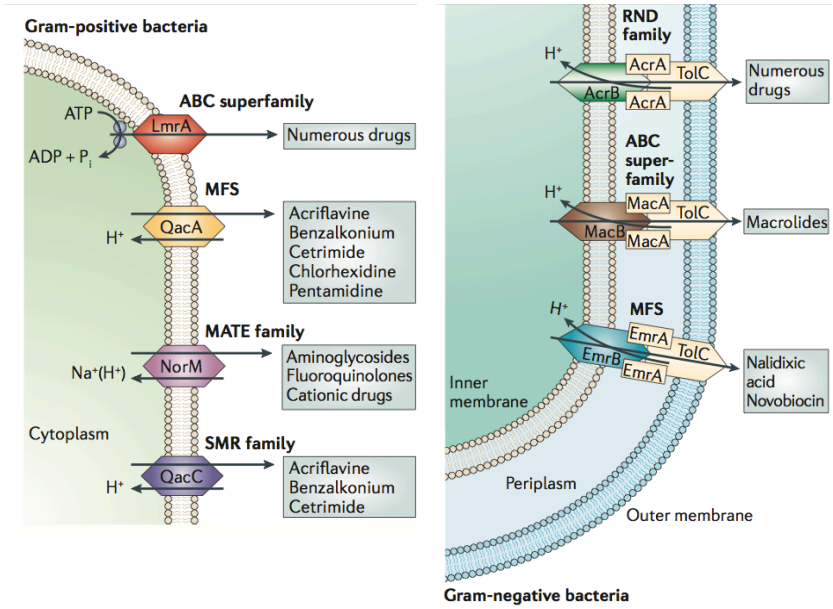

Fig. 8. Schematic representation of efflux pumps in Gram-positive and Gram-negative bacteria. Adapted from (78)

More information about drug efflux transporters from mycobacteria and *E. coli* will be detailed in chapters 1 and 3, respectively.

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Chapter 1

Role of MFS proteins in Drug Resistance and Virulence of *M. tuberculosis*

Introduction

Efflux pumps in mycobacteria have important roles

Drug-resistance

To prevent the intracellular accumulation of toxic compounds, bacteria have evolved energy-dependent systems to pump such molecules out of the cell. In mycobacteria, most of the drug resistant isolates have arisen through the acquisition of chromosomal mutations in genes encoding either drug targets or the drug activating enzymes (86), which usually confer high-levels of resistance. There are also a significant number of low-level drug resistant strains in which no mutation can be found. In these strains, the resistance phenotype can be explained in several ways: there could be mutations in other not yet discovered drug targets, alteration in the permeability of the cell envelope, or the resistance phenotype could be a consequence of the involvement of drug transporters, as it has been widely described for other bacterial genera (74).

Virulence

Transporters play an essential role in the expression of the virulence phenotype in bacteria. For example, AcrAB-TolC from *Salmonella enterica serovar Typhimurium* (22); regarding *M. tuberculosis*, the RND efflux pumps MmpL4, MmpL7, MmpL8 and MmpL10 are known to contribute to virulence (32, 37), as well as the ABC efflux pump DrrABC (23, 37).

Efflux pumps object of study

Rv1258c (Tap)

This efflux pump from *M. tuberculosis*, belonging to MFS, has 12 TMS (Fig 8). Rv1258c orthologue was first described in *Mycobacterium fortuitum*, a fast-growing mycobacteria causing diverse infections in humans as an efflux pump mediating low-level resistance to tetracycline, streptomycin, gentamicin and other aminoglycosides (3). The *M. tuberculosis* Rv1258c was characterised in heterologous hosts (*M. bovis* BCG and *M. smegmatis* mc^2155), showing a rather similar behaviour (3, 30, 82) . It was also observed that disruption of the Rv1258c-orthologue in the vaccine strain *M. bovis* BCG resulted in alterations in the growth kinetics (82). Other groups have revealed that this gene could be overexpressed in multi-drug resistant clinical isolates of *M. tuberculosis* and that its expression depends on the

transcription factor whiB7 (65). Besides, Rv1258c has been reported to be conferring rifampicin tolerance upon macrophage infection (1).

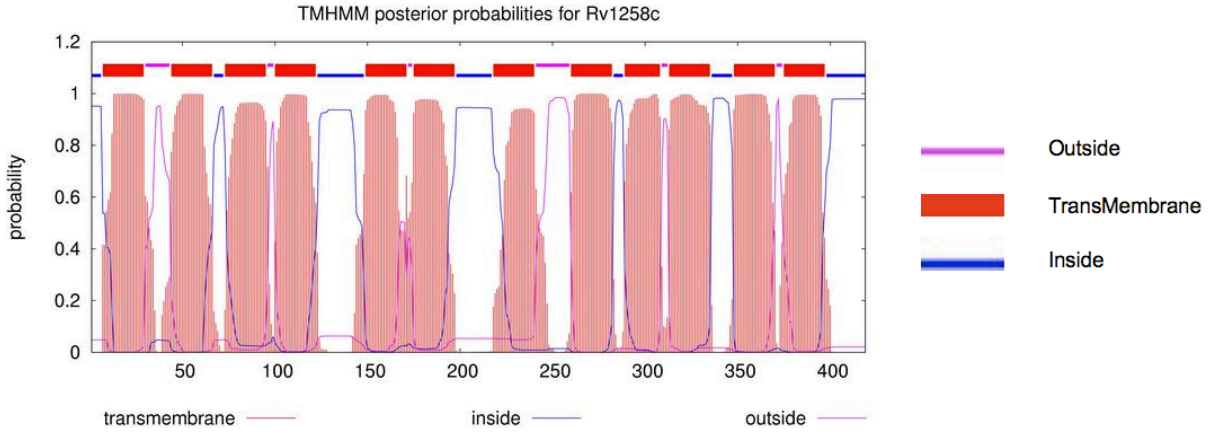


Fig .8. Prediction of TMS of Rv1258c efflux pump, using Hidden Markov Models (http://www.cbs.dtu.dk/services/TMHMM/)

Rv1410c (P55)

This efflux pump, a MFS protein with 12 TMS (Fig. 9) was first identified as the product of a gene downstream of that encoding the antigenic lipoprotein P27 from pathogenic M. bovis isolates in a two-gene operon (15, 16). Rv1410c efflux pump from *M. tuberculosis* was primarily characterised in the heterologous hosts *M. smegmatis* mc^2155 (90) and *M. bovis* BCG (81) and identified some of their drug-substrates, among which the antituberculous drug rifampicin seems to increase the expression of this gene in M. bovis BCG (following our results using lacZ as a reporter gene); in the latter microorganism, its inactivation also produced alterations in colony and bacillus morphology (82). A recent study clearly indicated that P27 (Rv1411c) and *Rv1410c* are functionally connected in processes that involve the preservation of the cell wall and the transport of toxic compounds away from the cells (11). Almost simultaneously, two groups described that insertional inactivation of the upstream *P27* gene resulted in strong attenuation of *M. tuberculosis* (17, 87); the first group also found a similar phenotype for the insertional inactivation of Rv1410c.

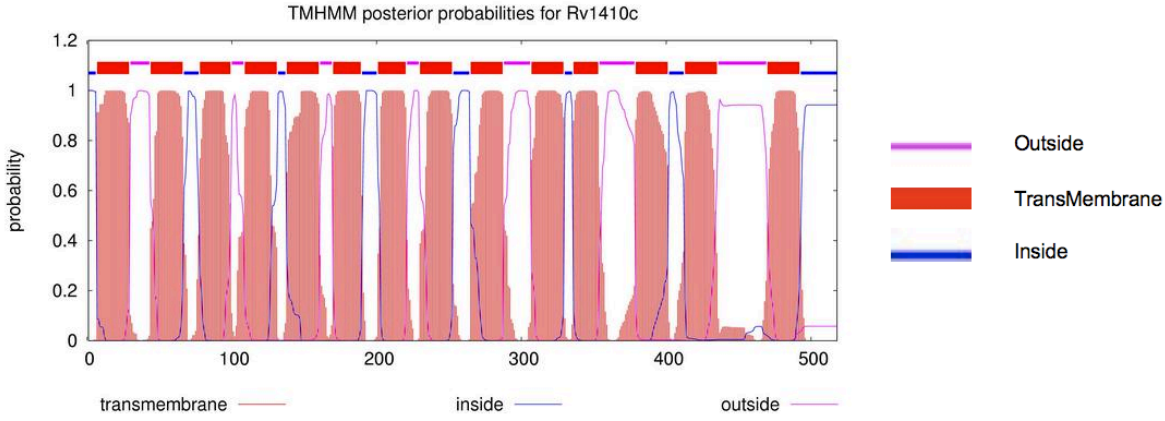


Fig .9. Prediction of TMS of Rv1410c efflux pump, using Hidden Markov Models (http://www.cbs.dtu.dk/services/TMHMM/)

Rv2333c (Stp)

It has been previously described the contribution of the Rv2333c efflux pump to intrinsic tetracycline and spectinomycin resistance of *M. bovis* BCG (80) where the inactivation of the gene resulted also in a slower growth rate in liquid media. Other group identified that the *M. tuberculosis* Rv2333c gene increased it expression by more than 10 times upon infection of human macrophages (25). Rv2333c protein belongs to the MFS Superfamily, and it has 14 TMS (Fig 10).

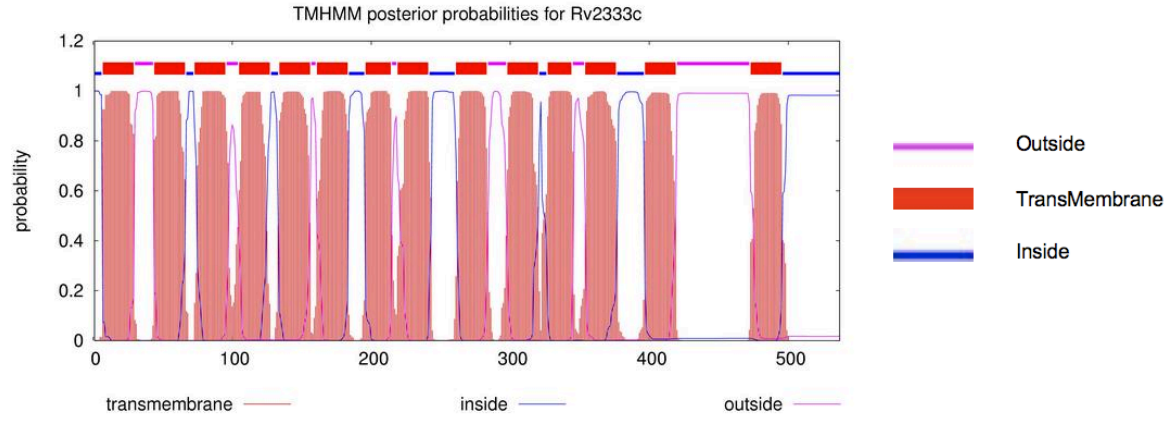


Fig. 10. Prediction of TMS of Rv1410c efflux pump, using Hidden Markov Models (http://www.cbs.dtu.dk/services/TMHMM/)

Genetic tools for generating knock-out strains in *M. tuberculosis*

The RecA protein was the first recombination enzyme to be identified in *M. tuberculosis*. As in *E. coli*, expression of *M. tuberculosis* RecA is associated with the SOS response that is triggered by DNA damage. Allelic exchange, or gene knockout, is an essential tool to investigate gene function in bacteria. It is usually achieved through the operation of homologous recombination systems to replace the target copy of the gene in the chromosome with an inactive copy of the gene located on a vector (88).

Initial attempts to achieve allele replacement in *M. tuberculosis* were frustrated by the high levels of illegitimate recombination in the pathogen (48). Numerous strategies have been devised to obtain efficient allele replacement in *M. tuberculosis*. Most experiments have utilized a plasmid delivery system, although phage systems have also been employed (73, 92). Most recently, a "recombineering" method was developed; it's based on the inducible expression of mycobacteriophage recombinases that enhance the frequency of recombination so that a linear fragment of DNA can recombinate in a single step (104).

OBJECTIVES

- To construct a knock-out, a complemented and an overexpression strain, derived from H37Rv virulent strain, for each of the efflux pump genes *Rv1258c*, *Rv1410c* and *Rv2333c*.

- To find if these efflux pump genes are implicated in intrinsic drug resistance of *M. tuberculosis*.

- To perform infection assays with these strains with the aim of finding a possible role of *M. tuberculosis* in virulence.

Material and Methods

Bacterial strains, media and growth conditions

In this work, we have used *M. tuberculosis* H37Rv and *E. coli* XL1, and *E. coli* DH5α.

E. coli strains were cultured in Luria-Bertani (LB) broth or on LB agar plates at 37ºC. Antibiotics were added when necessary, at final concentrations listed in Table 3. Liquid cultures were grown in glass tubes in a shaker.

Cultures and all manipulations of *M. tuberculosis* were done in a Biosafety Level 3 Laboratory (BSL3).

M. tuberculosis was cultured in Middlebrook 7H9 liquid medium supplemented with 10% ADC (0.5% bovine serum albumin, 0.2% dextrose, 0.085% NaCl, 0.0003% beef catalase) (Difco) (7H9/ADC from now). In order to keep the culture clump-free, Tween 80 was added to a final concentration of 0,05%, except for the media used in susceptibility assays and cultures for lipid extraction, which were supplemented with glycerol 0.5%.

Solid medium was Middlebrook 7H10 agar supplemented either with 10% ADC (7H10/ADC from now) or 10% OADC (0.05% oleic acid, 0.5% bovine serum albumin, 0.2% dextrose, 0.085% NaCl, 0.0003% beef catalase) (Difco) (7H10/OADC from now).

M. tuberculosis H37Rv and derivatives were grown at 37ºc; liquid cultures were done in cell culture flasks, without shaking.

Antibiotics, X-Gal (5-bromo-4-chloro-indolyl-ß-D-galactopyranoside) and sucrose were added when necessary at final concentrations listed in table 3. X-Gal was used to visualize the expression of the reporter gene *lacZ*, whereas sucrose was used for the counterselection of bacteria containing vectors carrying *sacB* gene.

Storage of strains was done at -80ºC in presence of 15% glycerol.

Compound	*E. coli*	*M. tuberculosis*
Kanamycin (Km)	20 µg/ml	20 µg/ml
Tetracyclin (Tet)	10 µg/ml	-
Hygromycin (Hyg)	50 µg/ml	50 µg/ml
X-GAL	64 µg/ml	64 µg/ml
Sucrose	-	2% (w/v)

Table 3. Antibiotics and other compounds used in this work.

General techniques of nucleic acids

DNA extraction

Genomic DNA extraction of mycobacteria

For DNA extraction the CTAB method was used (105). 1.5 ml of liquid culture was centrifuged (12000 rpm, 5 min) and the pellet resuspended in 400 µl of TE. This was heated for 10 min at 85ºC to inactivate the bacteria and 50µl of lysozyme 10mg/ml were added and incubated for minimum of 1 hour (but normally overnight) at 37ºC. Subsequently, 75 µl of the *Prot K/SDS* mix was added to each sample, and the suspension warmed for 10 min at 65ºC. After this, 100 µl of NaCl 5M and 100 µl of *CTAB/NaCl* pre warmed at 65ºC were added and samples incubated for further 10 min at 65ºC. Genomic DNA was extracted by adding 0.75 ml of chloroform:isoamyl alcohol 24:1. Samples were mixed by vortexing for 10 s before centrifugation (13000 rpm, 5 min). The aqueous phase was transferred to a fresh tube containing 420 µl of cold isopropanol, and samples were precipitated at -20ºC for at least 30 min. Nucleic acids were collected by centrifugation (13000 rpm, 10 min) and the pellets washed with 1 ml of cold EtOH 70%. After centrifugation (13000, 5 min) the pellet was vacuum dried, dissolved in 40 µl of double distilled water and treated with 1 µl of RNAse 1 mg/ml for 15 min. DNA was storaged at -20ºC.

DNA was quantified by absorbance readings at 260nm using a ND-1000 spectrophotometer (NanoDrop technologies).

Reagents & Solutions

CTAB/NaCl: 10% CTAB in 0.7 M NaCl

Prot K/SDS: 72.5 µl of SDS 10% + 2,5 µl Proteinase K 20 mg/ml

TE: 10 mM Tris HCl, 1 mM EDTA, pH8

Fast mycobacterial DNA extraction for PCR

This method releases plasmidic and genomic DNA from mycobacteria, usually in enough quantity to detect the presence of target genes by PCR. 500µl of liquid culture were centrifuged (12000 rpm, 5 min) and pellet resuspended in 400 µl TE. Alternatively, a colony can be disgregated in 400 µl TE. The bacterial suspensions were inactivated by heating at 90ºC for 30 min. After centrifugation (12000 rpm, 10 min), 350 µl of supernatant were transferred to a fresh tube and mixed with 350µl of chloroform:isoamyl alcohol 24:1 by vortexing for 10-20 sec. After centrifugation (12000 rpm, 5 min) the aqueous phase was transferred to a fresh tube; typically 2µl of this DNA solution were used in a standard PCR reaction.

Plasmid DNA extraction from Escherichia coli

Mini-preparation (Mini-prep)

1.5 ml of a liquid culture grown overnight were centrifuged (12000 rpm 15 min) and the pellet resuspended in 100 µl of *Solution I*. 200 µl of freshly made *Solution II* were added, and the content mixed by inverting the tube several times, until it becomes transparent and viscous. After incubation on ice for 5 min, 150 µl of cold *Solution III* were added and the tubes were mixed by inversion; as a result, a white pellet is formed. This mix was incubated on ice for 5 min, followed by centrifugation (12000 rpm 10 min). Supernatants were then transferred to a fresh tube and mixed with the same volumen of chloroform:isoamyl alcohol 24:1. After centrifugation, the aqueous phase was transferred to a tube containing 900 µl EtOH and 50 µl NaAc 3M and incubated at -20ºC for 30 min. By centrifugation (12000 rpm 5 min) small nucleic acids (plasmids and tRNA) were pelleted, and then washed with 100 µl EtOH 70%. Finally, the pellet was vacuum dried and resuspended in 20 µl double distilled water. RNA, which co-purifies with plasmidic DNA, was removed by adding 1 µl RNAse 1 mg/ml and incubating for 15 min at 37ºC. Plasmidic DNA was kept at -20ºC.

Reagents & Solutions

Solution I: 50 mM glucose, 10 mM EDTA, 25 mM Tris HCl pH8

Solution II: 0,2 M NaOH, 1% SDS

Solution III: 5 M NaAc, 11,5% Glacial HAc

TE: 10 mM Tris HCl, 1 mM EDTA, pH8

Maxi-preparation (Maxi-prep)

This method was used to obtain large quantities of plasmid. The process is the same as the mini-preparation, but starting with 100 ml of liquid culture. Larger volumes of solutions are needed: 5 ml *Solution I*, 10 ml *Solution II* and 7,5 ml *Solution III*. Besides, precipitation of plasmid DNA is done with isopropanol instead of ethanol. Finally, nucleic acids are disolved in 250 µl of double distilled water and treated with 10 µl RNase 1 mg/ml for 15 min at 37ºC.

Construction of plasmids

DNA fragments for cloning purposes were amplified by PCR using the *Pwo* high fidelity DNA polymerase (Roche), and gel-purified by using QIAquick Gel Extraction Kit (Qiagen). Plasmids were cut by restriction endonucleases and gel-purified by using QIAquick Gel Extraction Kit (Qiagen). Ligations were done using T4 DNA ligase (Invitrogen).

Construction of the replicative plasmid pCVZ1

A 3127 bp DNA fragment containing the operon *Rv1411c-P55* from *M. tuberculosis* H37Rv was amplified by PCR with oligonucleotides P55-C1 and P55-C2. This PCR fragment was digested with the restriction enzymes *Bgl*II and *Hind*III and inserted into pSUM36 replicative vector digested with *BamH*I and *Hind*III. To confirm the absence of PCR-induced mutations in the final plasmid pCVZ1 (Fig. 11), it was sequenced using the oligonucleotides M13Fw, M13Rv, cp55-1, cp55-2, p55-trans-in and vec19-up (Table 5).

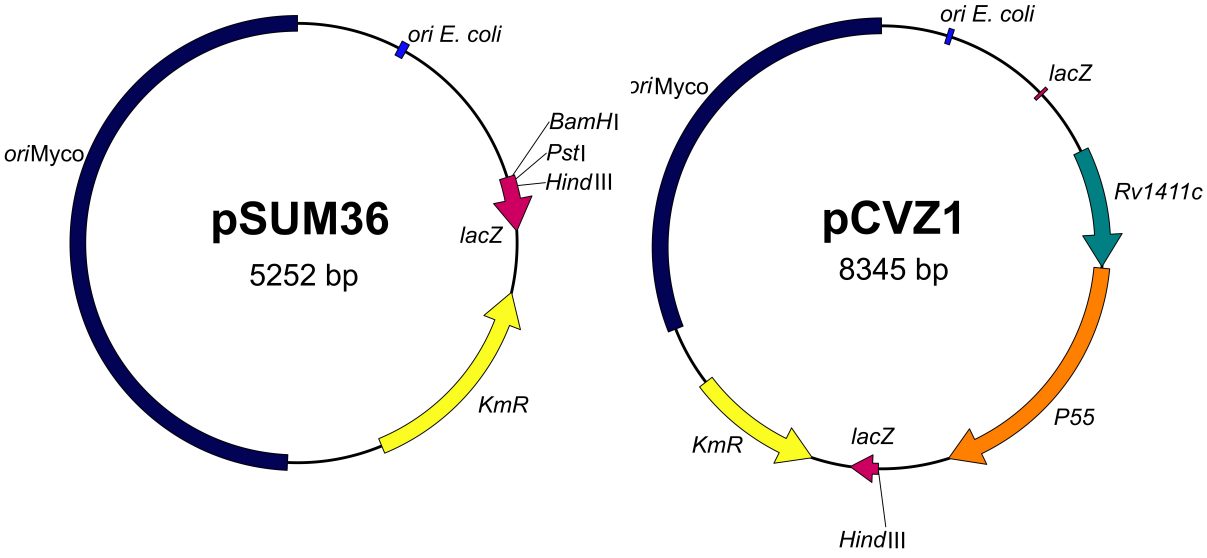


Fig 11. Replicative plasmid pSUM36 and its derivative, pCVZ1, carrying *M. tuberculosis* H37Rv *Rv1411c* and *P55* genes.

Construction of the integrative plasmids pCRS2, pCRS3 and pCRS4

All these plasmids were cloned in pMV361 vector, which has a multiple cloning site after the strong promoter P_{Hsp60}. During the cloning, we eliminated part of the P_{Hsp60} promoter by removing the fragment *Acl*I-*Hind*III of pMV361, then allowing the expression of the cloned genes under control of their own promoters.

In order to generate pCRS2 (Fig. 12), a 2494 bp DNA fragment containing *Rv2333c* (*stp*) gene from *M. tuberculosis* H37Rv was amplified by PCR with oligonucleotides Spt-C2 and Spt-C3. This PCR fragment was digested with restriction enzymes *Acl*I and *Hind*III and inserted into pMV361 integrative vector digested with *Acl*I and *Hind*III. The resulting plasmid was sequenced with oligonucleotides pMV361A, pMV361B, 2333-1F, 2333-1R, cspt1 and cspt2 (Table 5) to verify the sequence.

The plasmid pCRS4 (Fig. 12) was constructed by cloning *Rv1258c* (*tap*) gene from *M. tuberculosis* H37Rv into pMV361 vector. First, a 2201 bp DNA fragment containing *tap* gene was amplified by PCR with Tap-C2 and Tap-C3 oligonucleotides (Table 5). Following digestion of this PCR product with restriction enzymes, it was inserted in pMV361 vector previously linearized with *Acl*I and *Hind*III. Sequences obtained with oligonucleotides pMV361A, pMV361B, ctap5, ctap6, ctap7 and ctap8 were analysed to confirm the absence of mutations in the insert.

Plasmid pCRS3 (Fig. 12), carrying the operon *Rv1411c-P55* from *M. tuberculosis* H37Rv was generated in two steps. First, the fragment *Acl*I-*Pvu*II, containing part of P_{Hsp60}, was removed by digesting with restriction enzymes *Acl*I and *Pvu*II; the linearized vector was then treated with T4 DNA polymerase and recircularized, yielding pMVD361 (Fig. 12).

The second step consisted of an amplification of a 3127 bp DNA fragment containing the operon *Rv1411c-P55* from by PCR with primers P55-C2 and P55-C4, followed by digestion with the restriction enzymes *EcoR*I and *Hind*III. This fragment was inserted in pMVD361, also digested with *EcoR*I and *Hind*III, resulting in the plasmid pCRS3. Absence of mutations in the insert were checked by sequencing performed with oligonucleotides: pMV361A, pMV361B, cp55-1, cp55-2, p55-trans-in and vec19-up (Table 5).

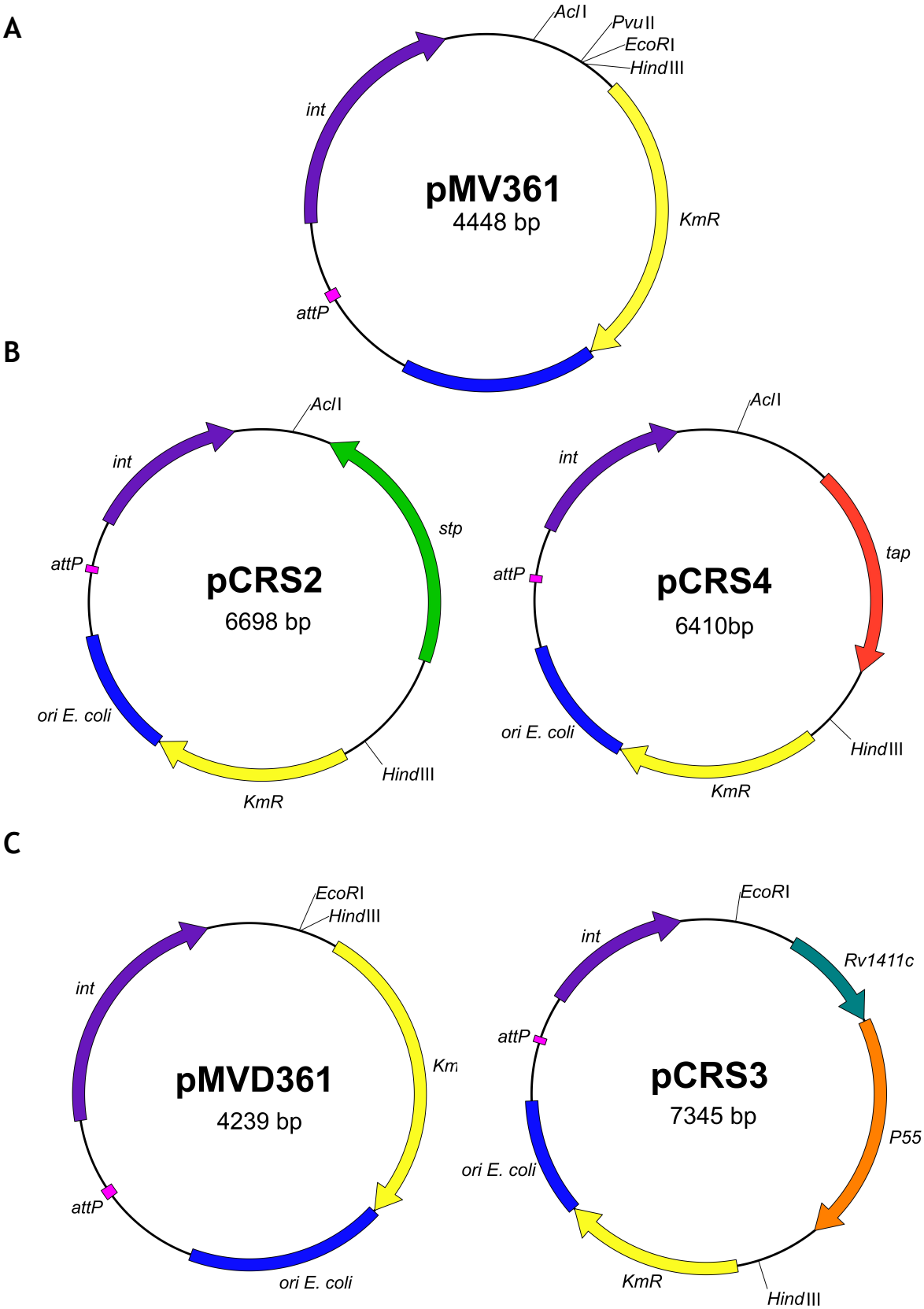


Fig 12. Integrative plasmid pMV361 and its derivatives. **A**. Vector pMV361 **B.** Plasmids pCRS2 and pCRS4 carrying *stp* and *tap* genes from *M. tuberculosis* H37Rv, respectively**. C**. Integrative plasmid pMVD361 and its derivative pCRS3, containing *Rv1411c* and *P55* genes from *M. tuberculosis* H37Rv.

These plasmids, along with the plasmids previously constructed by our group, are listed in Table 4.

Replicative plasmids			
Plasmid	**Marker genes**	**Description**	**Ref.**
pSUM36	*Km*^{R} *(*Tn5- derived)	Shuttle vector *E. coli/*mycobacteria for expressing genes in mycobacteria.	(4)
pPAZ11	*Km*^{R} *(Tn5-* derived)	pSUM36 derived plasmid with *Rv1258c (tap)* gene under control of its own promoter.	(82)
pSAN17	*Km*^{R} *(Tn5-* derived)	pSUM36 derived plasmid with *Rv2333c (stp)* gene under control of its own promoter.	(80)
pCVZ1	*Km*^{R} *(Tn5-* derived)	pSUM36 derived plasmid with *Rv1410c (P55)* gene under control of its own promoter.	This work
Suicide plasmids			
Plasmid	**Marker genes**	**Description**	**Ref.**
pVZ17	*Hyg*^{R}, *Km*^{R} (Tn903-derived), P_{Ag85}-*sacB*, P_{Hsp60}-*lacZ*	p2NIL/pGOAL derived plasmid, containing *Rv1258c (tap)* gene disrupted by a *Hyg*^{R} marker	(82)
pILI9	*Hyg*^{R}, *Km*^{R} (Tn903-derived), P_{Ag85}-*sacB*, P_{Hsp60}-*lacZ*	p2NIL/pGOAL derived plasmid, containing *Rv2333c (stp)* gene disrupted by a *Hyg*^{R} marker	
pILI12	*Hyg*^{R}, *Km*^{R} (Tn903-derived), P_{Ag85}-*sacB*, P_{Hsp60}-*lacZ*	p2NIL/pGOAL derived plasmid, containing *Rv1410c (P55)* gene disrupted by a *Hyg*^{R} marker	(81)
Integrative plasmids			
Plasmid	**Marker genes**	**Description**	**Ref.**
pMV361	*Km*^{R} (Tn903-derived)	Integrative vector, for expressing genes under control of Hsp60 promoter	(95)
pCRS4	*Km*^{R} (Tn903-derived)	pMV361 derived plasmid, containing *Rv1258c (tap)* gene under control of its own promoter.	This work
pCRS2	*Km*^{R} (Tn903-derived)	pMV361 derived plasmid, containing *Rv2333c (stp)* gene under control of its own promoter.	This work
pMVD361	*Km*^{R} (Tn903-derived)	pMV361 derived, lacking *Acl*I-*Pvu*II fragment	This work
pCRS3	*Km*^{R} (Tn903-derived)	pMVD361 derived plasmid, containing *Rv1410c (P55)* gene under control of its own promoter.	This work

Table 4. Plasmids used in this study.

Oligonucleotides

All the oligonucleotides are listed in Table 5. Artificial endonuclease restriction sites were added when required and are shown underlined.

Oligonucleotide	Sequence 5'->3'	Description
2333-1F	GACGGCCTGCAGTGGGTG	Sequencing pCRS2
2333-1R	CATGGCGACGGTCGTGATC	Sequencing pCRS2
2333-B	CCAGTGGAATTCGACGAAAC	To verify *stp* knock-out mutants.Sequencing.
2333-med	TCTGACCCTGTTCCGCG	To verify *stp* knock-out mutants.Sequencing.
cp55-1	TTGCTCACATGTTCTTTCCTG	Sequencing *P55* gene
cp55-2	CGACGGCAAACACGTACTG	Sequencing *P55* gene
cspt1	GGTGAGCACCGCGATTG	Sequencing pCRS2
cspt2	GTTCCTGCAGAACGTGCG	Sequencing pCRS2
ctap5	GTCGACTACTTCGGGCGTC	To verify *tap* knock-out mutants.Sequencing.
ctap6	GACCATCGATGCCAAACC	To verify *tap* knock-out mutants.Sequencing.
ctap7	GTCCAACGACCAGCCTGC	Sequencing *tap* gene.
ctap8	CGCTGTATCTGCCGATGG	Sequencing *tap* gene.
Hyg-out	TGATCCGGTGGATGACC	To verify knock-out mutants
Km903-A	CTCGTGAAGAAGGTGTTGCT	To confirm presence of Km^R Tn903- derived gene
Km903-B	CCGACCATCAAGCATTTTAT	To confirm presence of Km^R Tn903- derived gene
M13Fw	GTAAAACGACGGCCAGT	To sequence pSUM36 and derivatives
M13Rv	AGCGGATAACAATTTCACAC	To sequence pSUM36 and derivatives
P55 trans-in	ACGCCCTGGCCGAACAGC	To verify *P55* knock-out mutants.Sequencing.
P55-C1	TTTTAGATCTTTCACCGGTGGCGTCC	To clone *P55* gene in pSUM36. *Bgl*II site underlined.
P55-C2	TTTTAAGCTTCTTGGTCGGCACCGGC	To clone *P55* gene in pSUM36 and pMVD361. *Hind*III site underlined.
P55-C4	TTTTGAATTCTTTCACCGGTGGCGTCC	To clone *P55* gene in pMVD361 *EcoRI* site underlined.
p55-out1	GGATGACCGGCATGTTGATC	To confirm presence of pCRS3
pks3-Fw	CGCTGACGTCGGTGAAAAC	To sequence position 1467 of *pks3* gene
pks3-Rv	CTGCACCCGGTCAATACC	To sequence position 1467 of *pks3* gene
pMV361-A	CAGGAGCATTGCCGTTCC	Sequencing pMV361 and derivatives
pMV361-B	CCTCGAGCAAGACGTTTCC	Sequencing pMV361 and derivatives
RP-180	ATGCAGCTGGCACGACAGGT	To verify replicative plasmids derived from pSUM36.
Spt-C2	CCAACAACGTTTTGGCGTTTCC	To clone *stp* gene in pMV3*61. Acl*I site underlined.
Spt-C3	CTGCAGCCAAGCTTGCATGCC	To clone *stp* gene in pMV3*61. Hind*III site underlined.
spt-out1	CCAGTGGCGATGAGCGTGAG	To confirm presence of pSAN17and pCRS2
Tap-C2	TTTTAACGTTGCCCGGGGGCGCAC	To clone *tap* gene in pMV3*61 Acl*I site underlined.
Tap-C3	TTTTAAGCTTGTACAGGCCGGGCTGGC	To clone *tap* gene in pMV3*61 Hind*III site underlined.
tap-out1	CAGCGTTGCGAACAGGATCAG	To confirm presence of pPAZ11 and pCRS4
TN5-A	CGCTTGGGTGGAGAGGCTATTC	To confirm presence of Km^R Tn5- derived gene
TN5-B	CCGCTCAGAAGAACTCGTCAAG	To confirm presence of Km^R Tn5- derived gene
vec19-up	AGCAGGACGTCGAGTCGCG	To verify *P55* knock-out mutants.Sequencing.

Table 5. Oligonucleotides used in this study.

Southern Blot

Approximately 3 µg of extracted genomic DNA were digested with *Pvu*II or *Pst*I (see table x). The fragments were separated by electrophoresis through 0.8% agarose gels in Tris-Borate-EDTA (TBE) buffer for 18h at 36V. After migration, a standard treatment of the gel was performed. DNA was fixed by UV light for 5min and subjected to a depurination treatment (0.25M HCl for 10min), washing, denaturalization (0.5M NaOH, 1.5M NaCl for 20min) and neutralization (1.5M NaCl, 1M Tris, pH=8.0 for 20min). Hereafter, DNA was vacuum blotted (45-60mbar for 1h 30min) onto a Hybond-N+ nylon membrane (Amersham) using 10x SSC buffer (1.5M NaCl, 150mM sodium citrate). Blotted DNA was fixed by UV light for 4 min and hybridized with a specific probe. This probe was made by PCR with Taq Gold polymerase (Applied Biosystems) using specific oligonucleotides (Table 6). This PCR product was then purified using GFXTM PCR kit and adjusted to 10 ng/µl. After labeling the probe, hybridization patterns were visualized using ECLTM Direct Nucleic Acid Labelling and Detection System (Amersham).

M. tuberculosis **H37Rv deleted gene**	**DNA digested with**	**Probe**	
		PCR with oligonucleotides	**Size**
Rv1258c	*Pst*I	ctap5 vs ctap6	0,7 Kb
Rv2333c	*Pvu*II	2333-med vs 2333-B	1,1 Kb
Rv1410c	*Pvu*II	p55-trans-in vs vec19-up	0,9 Kb

Table 6. Restriction enzymes used for digesting genomic DNA and construction of specific probes for Southern Blot.

Generation of competent cells & electroporation

E. coli

To prepare electrocompetent cells, 300 ml of a bacterial culture were grown to an OD_{600nm} of 0.4 - 0.6. Then the growth was stopped for 30 min on ice, and bacteria were washed twice in chilled-cold water, and once in chilled-cold 10% glycerol. Cells are finally resuspended in 1 ml chilled-cold 10% glycerol. Aliquots of 40 µl can be storaged at -80ºC for further use.

Aliquots of 40 µl were electroporated with ~100 ng purified plasmid DNA in 0.2 cm gap cuvettes (Bio-Rad) with a single pulse (2.5kV, 25µF, 200Ω) in a GenePulser XcellTM (Bio-Rad). Cells were resuspended in LB to a final volume of 1 ml and incubated for 1h at 37ºC

before plating several dilutions on plates containing the needed antiobiotic. Colonies appeared after incubation overnight.

M. tuberculosis

M. tuberculosis competent cells were prepared as described by Wards *et al*. (108). 200 ml of bacterial culture were grown to an OD_{600nm} of 0.6-0.8. 24 h before preparing the competent cells, glycine was added to the cells to a final concentration of 0,2 M and incubated at 37ºC.

All the process was performed at room temperature. Bacterial pellet was washed twice with 0.05% Tween- 80 and once with 10% glycerol-0.05% Tween-80, and finally resuspended in 2ml of 10% glycerol-0.05% Tween-80. Aliquots of 200-400 µl of competent cells can be storaged at -80ºC for further use.

Aliquots of 200-400µl were electroporated with 100-200 ng of replicative or integrative plasmid DNA (previously purified), using 0.2 cm gap cuvettes (Bio-Rad) with a single pulse (2.5kV, 25µF, 1000Ω) in a GenePulser XcellTM (Bio-Rad). Cells were recovered with 1ml of 7H9-ADC-0.05% Tween-80 and incubated for 24h at 37ºC, to express the antibiotic resistance genes, before plating serial decimal dilutions on plates containing the relevant antibiotic. Colonies typically appeared in 3-4 weeks.

Suicide plasmids

Electrocompetent cells were prepared the same way, but resuspending in a final volume of 1,5 ml 10% glycerol-0.05% Tween-80 in order to obtain a denser cell suspension. Fresh 400 µl aliquots were transformed with 15-20 µg of purified plasmid DNA, which had been previously irradiated with UV light (λ=360nm) for 2-10 s. All the transformation mix was plated, dividing it in three agar plates. Colonies typically appeared in 4-5 weeks.

Construction of knock-out strains of *M. tuberculosis*

Knock-out mutants of *M. tuberculosis* H37Rv were constructed following the method described by *Parish et al*. (73). A suicide plasmid fulfilling several characteristics is needed (Fig 13).

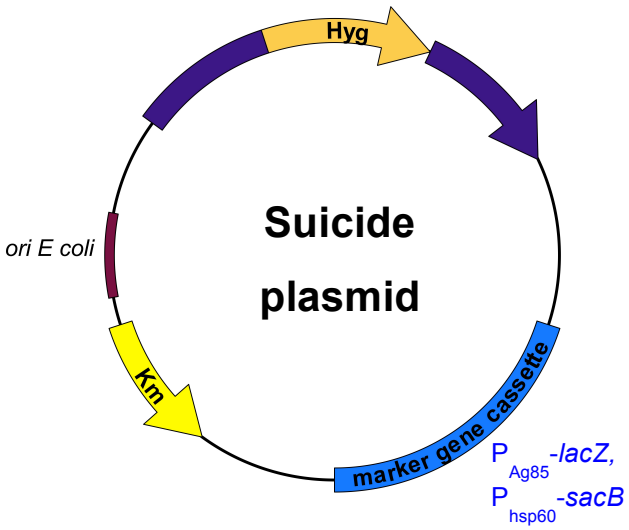


Fig. 13. Scheme of suicide plasmids for generating knock-out mutants.

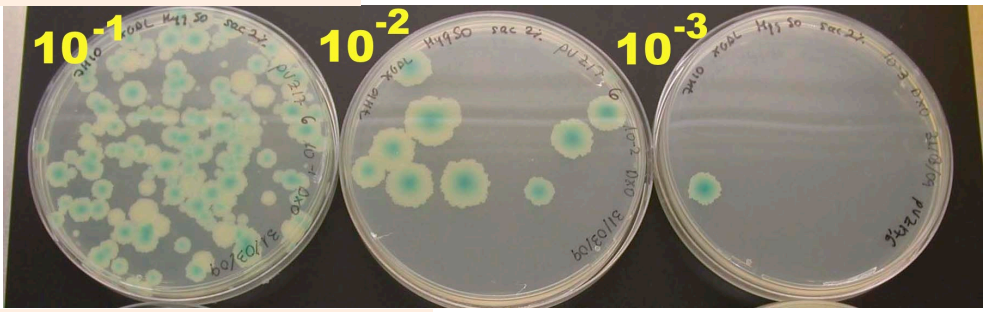
This plasmid has the target gene disrupted by a *Hyg* resistance marker, in addition to a *Km* resistance marker and a cassette containing the genes *lacZ* and *sacB* under control of the strong promoters P_{Ag85} and P_{hsp60} respectively. Since it lacks an origin of replication for mycobacteria, it is a suicide plasmid.

A suicide plasmid derived from pNIL and pGOAL series had been previously constructed in our group for each target gene *Rv1258c (*pVZ17*)*, *Rv2333c* (pILI9) and *Rv1410c* (pILI12) (see *plasmids*).

When this plasmid was electroporated in *M. tuberculosis (*see *Generation of competent cells and electroporation, M. tuberculosis, suicide plasmids)*, a first event of simple recombination can take place between the disrupted gene of the plasmid and the wild-type gene in the genomic DNA resulting in a recombinant strain, named Single Cross Over (SXO) mutant. SXO strain has the suicide plasmid integrated in its genomic DNA, so mutants are resistant to Hyg and Km, and have a lactose positive phenotype; for this reason they were selected in 7H10/ADC supplemented with Hyg, Km and X-Gal. Blue colonies (lac^+) were candidates for being SXO. Thus, they were inoculated in 7H9/ADC with Hyg and Km, and incubated for 10-15 days at 37ºC. Several PCRs were performed to verify this first recombination.

In order to induce the second recombination event, several decimal dilutions (10^{-1}, 10^{-2} and 10^{-3}) of SXO strain liquid culture were plated on 7H10/ADC supplemented with Hyg, X-Gal and sucrose. The marker gene cassette has the counter-selection marker *sacB,* which is lethal for bacteria in the presence of sucrose (75). Thus, SXO mutant wasn't able to grow in the selection medium, except the case when the wild-type and the disrupted genes recombinated, removing the plasmid from the genome. This second recombination event yielded the Double Cross Over (DXO) mutant, which was the final knock-out strain, with a phenotype Hyg and sucrose resistant, Km sensitive and lactose negative. White colonies (lac^-) were candidates for being DXO strains, so they were inoculated in 7H9/ADC/Hyg and cultured for 10-15 days, then checked by PCR and Southern Blot

A. Medium with sucrose



B. Medium without sucrose

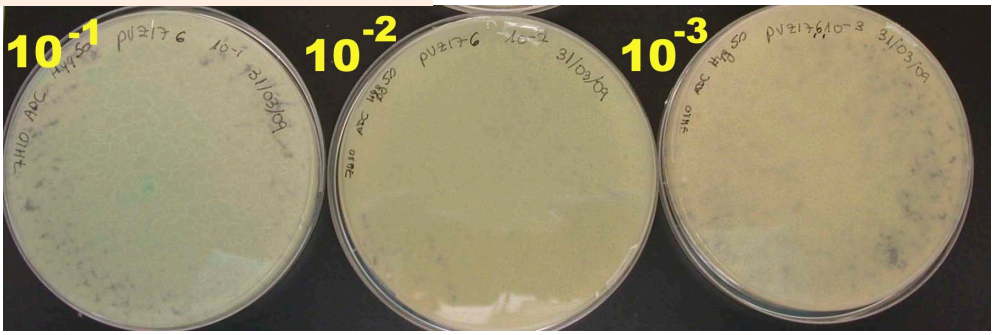


Fig. 14. Comparison of growth of *M. tuberculosis* carrying *sacB* gene in medium with and without sucrose. Dilution of the SXO liquid culture is indicated. White colonies (lac^-) are candidates for DXO mutants, whereas SXO are spontaneous mutants resistant to sucrose.

Phenotypical characterization of *M. tuberculosis*

Growth in liquid medium

In order to characterize growth in liquid medium, 100 ml of 7H9/Tween/ADC were inoculating with 10^5 cfu/ml, using a culture in logarithmic phase. OD_{600nm} was measured for 1 month. When OD_{600} reached 1, the culture was diluted ½ and ¼ and OD corrected depending on the dilution.

Neutral Red Stain

This method was adapted from *Soto et al*. (93). Mycobacterial strains were grown on 7H10/ADC medium for 3-4 weeks. Cells were placed and gently disgregated in Falcon 15ml tubes containing 4ml of *50% methanol*, and then incubated for 1h at 37ºC. Cells were pelleted by centrifugation (4000 rpm, 5 min), and methanol removed. Then, 4ml *barbital buffer* were added and the pellet mixed by inversion. Subsequently, 150µl of a solution of *neutral red* were added. Results were evaluated after 1h incubation at 37ºC.

Reagents & Solutions

50% methanol: 50% methanol, 50% distilled water

Barbital buffer: 1% sodium barbital in 5% NaCl, pH=9.8

Neutral red: 0,05% neutral red disolved in distilled water.

Analysis of lipids of *M. tuberculosis* cell wall

Extraction of free lipids and mycolic acids from *M. tuberculosis* cell wall

Strains were inoculated in 200 ml 7H9/ADC/0,5% glycerol, it is important not to add Tween 80 because the free lipids can be partially lost. After approximately one month of incubation at 37ºC, cultures were centrifuged (4000 rmp, 20 min) and PBS 1x added to wash the cells. Subsequently, cells were pelleted (4000 rpm, 1h) and PBS removed by decanting. Pellets can be frozen and storaged at -80ºC or used immediately.

First extraction was performed with chloroform:methanol 1:1. Bacterial pellets were let to dry as much as possible before resuspending them in 4 ml methanol. The suspension was then transferred to a glass tube with PTFE cap and 4 ml chloroform were added. This mix was incubated for 2 days at room temperature, gently shaking occasionally. The organic phase containing free lipids was then transferred to a fresh glass tube.

A second extraction was made using 4 ml chloroform:methanol 2:1. The two extracted fractions were joined in the same glass tube, and further evaporation of the organic phase with a stream of nitrogen gas resulted in dried pellet of lipids.

The remaining bacterial residues were kept to extract mycolic acids. First, 8 ml of MeOH:toluene:H_2SO_4 (30:15:1) were added to the glass tube containing the bacterial residues, and heated at 80ºC overnight, vortexing occasionally. In this process the esther bond was broken and mycolic acids released as methylic esthers. Two consecutive extractions with 8 ml hexane were made, and hexane further evaporated with a stream of nitrogen gas.

Thin Layer Chromatography

Crude extracts and mycolic acids were resuspended in 100 µl and 200 µl chloroform, respectively. 10 µl of each extract were deposed on silica gel G60 plates (20x20cm; Merck)

and analyzed by thin layer chromatography (TLC). As controls, 10 µl of purified fractions of *M. tuberculosis* cell wall free lipids (2mg/ml) were included.

Different solvents were used depending on the polarity of the lipids we want to separate (Table 7). To visualize lipids, several reagents can be used: *anthrone* for glycolipids, Dittmer (molybdenum blue) for phospholipids and *molybdophosphoric acid* (MPA) for general lipids. Plates were sprayed with the chosen reagent and then heated a 110ºC-120ºC until coloured spots appeared.

Lipids	Eluent (v:v)	Reagent
PAT, DAT, CF, SL-I	$CHCl_3$:MeOH 85:15	Anthrone
PIMs	$CHCl_3$:MeOH:H_2O 60:35:8	Anthrone
Phospholipids	$CHCl_3$:MeOH:H_2O 60:35:8	Dittmer
PDIM, TG	Petroleum ether:diethylether 90:10	PMA
PGLs	$CHCl_3$:MeOH 95:5	Anthrone
Mycolic acids	Hexane: diethylether 85:15	PMA

Table 7. Eluents and Reagents used in TLC for each group of lipids.

Reagents & Solutions

Anthrone: 1% anthrone in H_2SO_4

Molybdophosphoric acid: molybdophosphoric acid 10% in ethanol

Susceptibility assays: Minimum Inhibitory Concentration

The resazurin assay

Serial two-fold dilutions of antibiotics were performed in 7H9/glycerol 0,5%/ADC medium, in 96-well microtiter plates, with a final volume of 100µl per well.

Liquid cultures of *M. tuberculosis* in logarithmic phase were adjusted to 10^5 cfu/ml in 7H9/glicerol 0,5%/ADC. 100µl of this suspension were added to each well and plates were incubated 6 days at 37ºC. 30 µl of *resazurin* solution were then added to each well, and results were observed after 48h of incubation at 37ºC. Resazurin (blue) is an indicator of bacterial growth, since metabolic activity of bacteria reduces it to resofurin (pink).

Minimum Inhibitory Concentration (MIC) is the concentration of antibiotic of the first well that doesn't change color from blue to pink.

Reagents & Solutions

Resazurin: 0,1 mg/ml resazurin in distilled water

Infection assays

Intracellular replication in J774 mouse macrophages

Intracellular replication of *M. tuberculosis* was tested in J774 mouse macrophage-like cells. J774 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 4mM L-glutamine ("complete DMEM" from now). Infections were performed at a multiplicity of infection (MOI) of 0.5-1 bacteria per macrophage, with a variable number of cells per well depending on the experiment. After 4 h, infection was stopped by removing bacterial suspension and cells were washed three times with 1 ml PBS, and finally cultured in 2ml complete DMEM per well to allow intracellular replication. Medium was replaced when needed (typically every 1-2 days) by removing 1,5 ml of overlaying DMEM and adding the same volume of fresh complete DMEM.
In order to know the number of intracellular bacteria at the end of time of infection (4h) and days 3, 5 and 7 post-infection, DMEM medium was removed, and 300 µl 0.1% triton X-100 were added to each well in order to lyse the cells. 700 µl of PBS were added per well and the solution carefully mixed. Several dilutions of this lysate were plated on 7H10/ADC plates and viable bacteria counted after 2-4 weeks.

Apoptosis induction in J774 mouse macrophages

Apoptosis induction of *M. tuberculosis* was tested in J774 mouse macrophage-like cells. J774 cells were seeded in wells, in complete DMEM. Infections were performed at a multiplicity of infection (MOI) of 30-50 bacteria per macrophage, with 10^5 cells per well. After 4 h, infection was stopped by removing bacterial suspension and cells were washed three times with 1 ml PBS, and finally cultured in 2ml complete DMEM per well to allow intracellular replication.

At day 3 or 4 post-infection, both supernatant and trypsinized cells were collected together in 15 ml screw-cup tubes. Phosphatidylserine exposure and membrane integrity were analyzed by using Annexin-V and 7-actinomycinD (BD Biosciences) and flow cytometry according to manufacturer instructions. Briefly, cells were washed with PBS and incubated

with Annexin-V and 7AAD in Annexin-binding buffer for 15 min. After that, cells were washed twice with PBS, fixed with 4% paraformaldehyde (PFA) during 30 min and washed again with PBS. Both PBS and PFA solutions contained CaCl2 2.5 mM.

Replication in lungs in mouse model

Intratracheal infection in C57BL/6 mice was performed with 150-300 CFU of bacteria in 50µl of PBS 1x per mouse and 5 mice were used per strain. To deliver bacterial suspension, isoflurane anesthetized mice were orally intubated with a lachrymal olive luer-lock (UNIMED), 30mm in length and 0.6mm in diameter. Serial dilutions of the bacterial suspension were plated to corroborate the number of viable bacteria. Three weeks post-infection, lungs from each animal were harvested and placed in PBS for bacterial burden evaluation. To analyze bacterial replication, lungs were homogenized using GentleMacs homogeneizer (Miltenyi Biotec) and CFU counted by plating serial dilutions on 7H11/ADC solid medium supplemented with polymyxin 50U/ml, trimethopim 20mg/l and amphotericin B 10mg/l.

The protocol for animal handling was previously approved by University of Zaragoza Animal Ethics Committee.

Results and Discussion

Generation of *M. tuberculosis* knock out mutants

We have used the method described by *Parish et al.* (73) to generate three knock-out mutants, H37Rv KOTAP, H37Rv KOSTP and H37Rv KOP55, with *Rv1258c*, *Rv2333c* and *Rv1410c* genes deleted respectively.

The correspondent suicide plasmid (Table 8) was electroporated in *M. tuberculosis* H37Rv competent cells, and the colonies grown on 7H10/ADC/Hyg/X-Gal that presented lactose positive (blue) phenotype, selected as potential SXO candidates. These colonies were grown in liquid medium, and subsequently verified by PCR (Table 9).

Suicide plasmid	Targeted gene	*Knock-out* strain
pVZ17	*Rv1258c*	H37Rv KOTAP
pILI9	*Rv2333c*	H37Rv KOSTP
pILI12	*Rv1410c*	H37Rv KOP55

Table 8. Suicide plasmids used to generate H37Rv efflux pumps knock-out mutants.

The second event of recombination, which will yield the knock-out mutant, was induced by inoculating the SXO strains on 7H10/ADC/Hyg/suc plates. After 4 weeks, the colonies with Hyg resistant and lactose negative (white) phenotype were inoculated in liquid medium, as they were candidates for DXO mutants. Most of the colonies were lactose positive; they were spontaneous mutants to sucrose. Several PCRs were performed to confirm the DXO candidates (Table 9). The whole process of knock-out generation took about 6 months.

Rv1258c	**Size of PCR product (Kb)**		
PCR	**Wild-type**	**SXO**	**DXO**
ctap5 *vs* Hyg-out	---	0.5	0.5
Hyg-out *vs* ctap6	---	0.5	0.5
ctap5 *vs* ctap6		0.7 + 3	3
Km903-A vs Km903-B	---	1.5	---
Rv2333c	**Size of PCR product (Kb)**		
PCR	**Wild-type**	**SXO**	**DXO**
2333B *vs* Hyg-out	---	1	1
Hyg-out *vs* 2333-med	---	0.4	0.4
2333B *vs* 2333-med		1.1 + 3	
Km903-A vs Km903-B	---	1.5	---
Rv1410c	**Size of PCR product (Kb)**		
PCR	**Wild-type**	**SXO**	**DXO**
vec19-up *vs* Hyg-out	---	0.6	0.6
Hyg-out *vs* P55-trans-in	---	0.6	0.6
vec19-up *vs* P55-trans-in	0.9	0.9 + 3.2	3.2
Km903-A vs Km903-B	---	1.5	---

Table 9. PCRs for confirming SXO and DXO mutants, for the efflux pump genes *Rv1258c*, *Rv2333c* and *Rv1410c*.

Confirmation of knock-out mutants by Southern Blot

Finally, Southern Blot was the definitive test to validate the knock-out mutants (Fig 15-17). For this, genomic DNA was digested with a restriction enzyme that has little number of recognition sites in the genome, generating fragments of different sizes. For each knock-out strain, we chose a restriction enzyme that yielded each efflux pump gene in a separate fragment. A specific probe was then hybridazed, revealing the fragments that contained the efflux pump genes, either wild-type or disrupted by the *Ω-Hyg* cassette. Both the wild-type and the DXO mutant had only one band each, corresponding to the wild type and the disrupted gene, respectively. The SXO presented always two bands, one containig the wild type and the other containing the disrupted gene; in addition, depending on how the suicide plasmid recombinated, two different SXO strains could appear.

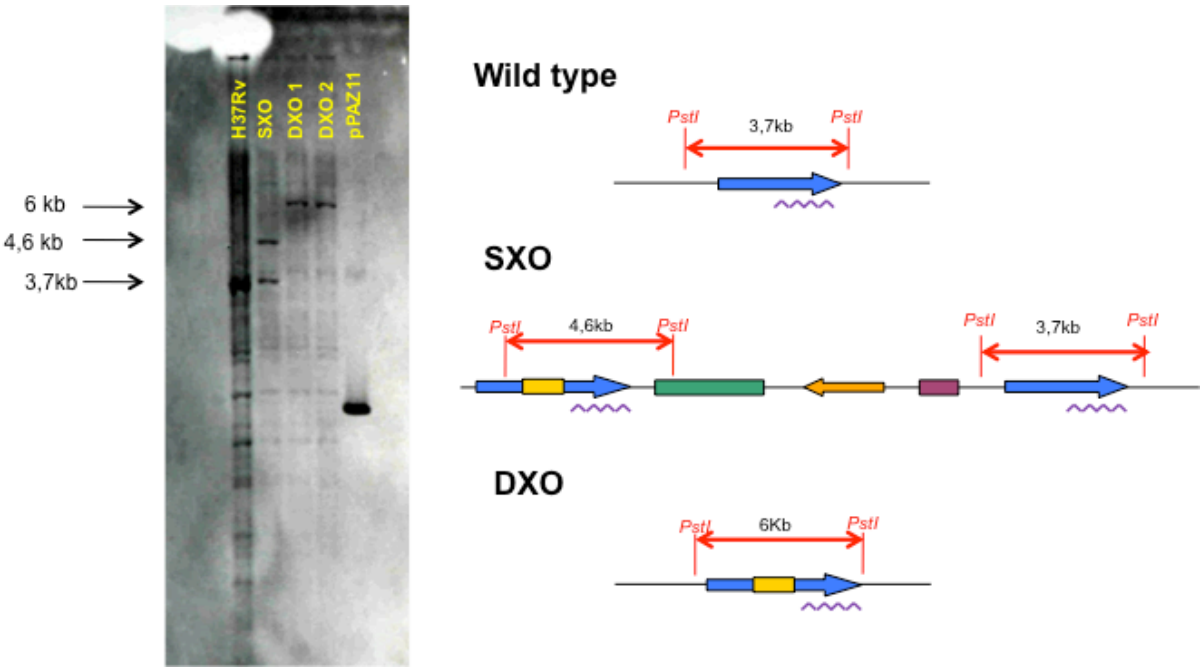


Fig 15. Southern Blot of *Rv1258c* (*tap*) SXO and DXO strains. pPAZ11 digested with *Pst*I was used as control of hybridization.

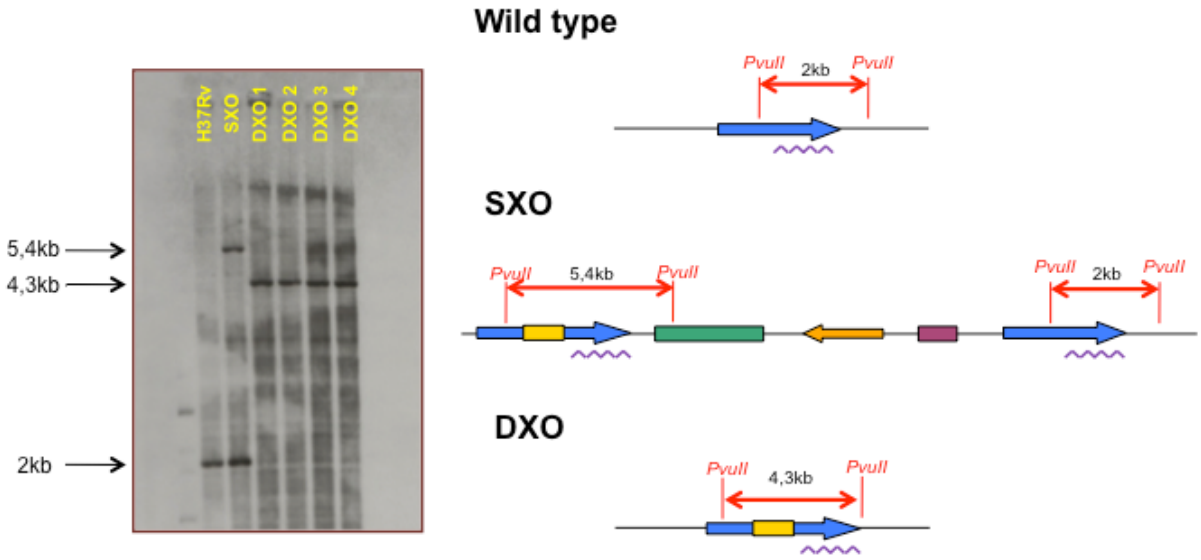


Fig. 16. Southern Blot of *Rv2333c* (s*tp*) SXO and DXO strains.

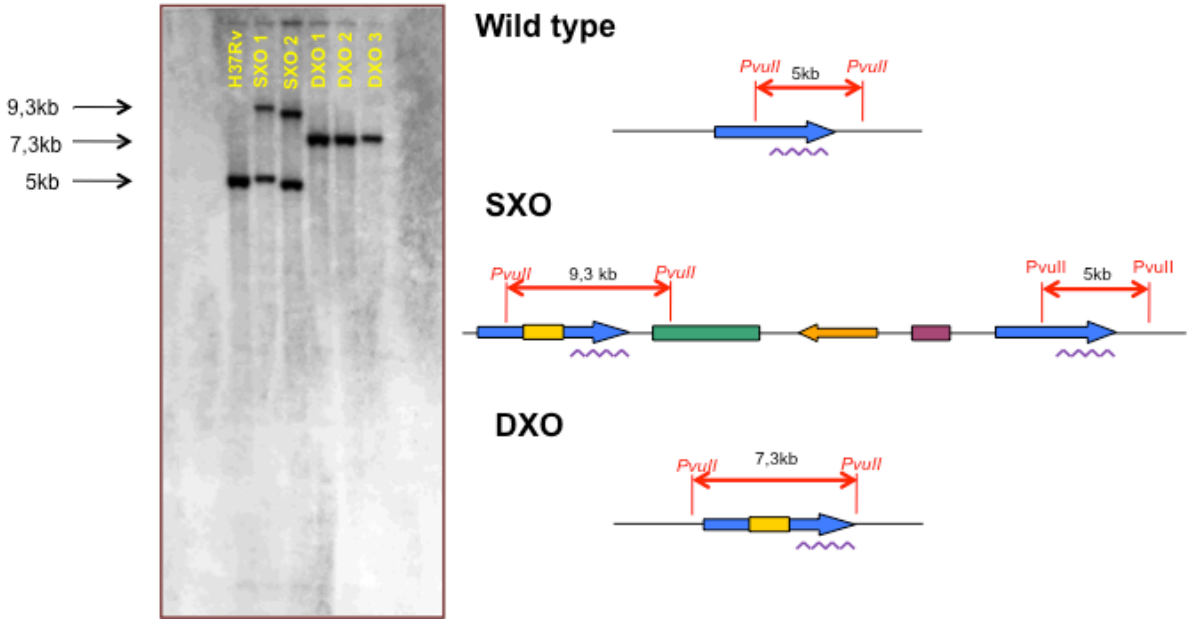


Fig. 17. Southern Blot of *Rv1410c* (*P55*) SXO and DXO strains.

Generation of complementation and overexpression strains

With the aim of constructing these strains, two kind of plasmids were obtained for each efflux pump gene:

- Replicative plasmids, containing an efflux pump gene under control of its own promoter. Plasmids pPAZ11, pSAN17 and pCVZ1 were electroporated in H37Rv, in order to generate overexpression strains. All the transformant strains were verified by PCR (Table 10).

- Integrative plasmids, with an efflux pump gene under control of its own promoter. Plasmids pCRS4, pCRS2 and pCRS3 were electroporated in their respective knock-out mutant to obtain the complemented strains. All the transformant strains were verified by PCR (Table 11).

Overexpression strains			
Strain	**Description**	**PCR verification**	
		Oligonucleotides	**Size of PCR product (Kb)**
H37Rv pPAZ11	Overexpression strain for Rv1258c (Tap)	RP-180 *vs* tap-out1	0,9
		TN5-A *vs* TN5-B	0,8
H37Rv pSAN17	Overexpression strain for Rv2333c (Stp)	RP-180 *vs* spt-out1	0,8
		TN5-A *vs* TN5-B	0,8
H37Rv pCVZ1	Overexpression strain for Rv1410c (P55)	TN5-A *vs* TN5-B	0,8

Table 10. PCRs for verification of efflux pump overexpression strains.

Complemented strains			
Strain	**Description**	**PCR verification**	
		Oligonucleotides	**Size of PCR product (Kb)**
H37Rv KOTAPc	H37Rv KOTAP with pCRS4	pMV361A *vs* tap-out	0,9
H37Rv KOSTPc	H37Rv KOSTP with pCRS2	pMV361B *vs* stp-out	1
H37Rv KOP55c	H37Rv KOP55 with pCRS3	pMV361B *vs* p55-out	1

Table 11. PCRs for verification of complemented knock-out mutants.

In summary, for each efflux pump (Rv1258c, Rv2333c and Rv1410c) four H37Rv derivative strains have been generated: i) SXO mutant, ii) DXO mutant, iii) the complemented knock-out strain, and iii) the overexpression strain. All these strains were inoculated in 7H10/ADC/X-Gal with several combinations of Hyg and Km, as a microbiological test, following the scheme shown in Fig. 18.

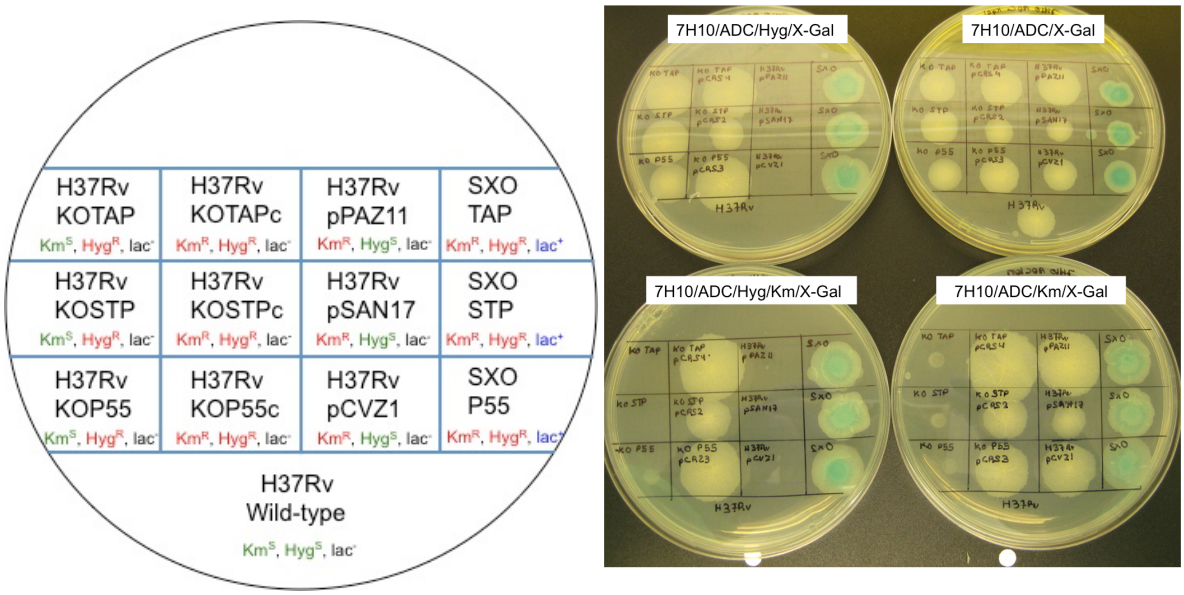


Fig. 18. Microbiological test of the efflux pumps mutant strains. **A**. Points of inoculation of each strain; phenotype is indicated. **B**. Result of the test, strains grew or not according to their phenotype.

Phenotypical characterization of *M. tuberculosis* efflux pumps mutants

The generated strains were Neutral Red positive

Neutral red stain is a fast screening method for testing integrity of the mycobacterial cell envelope, because neutral red phenotype seems to be consequence of the absence of more than one type of methyl-branched fatty acids (26). Two strains were used as controls (Fig 19): H37Rv, a virulent strain, as positive control; and MTBVAC, an attenuated strain, as negative control. MTBVAC, which has double unmarked *phoP* and *fadD26* deletions, is not able to fix neutral red because the *fadD26* gene is required for PDIM biosynthesis (91).

Since the generated strains were going to be tested in infection assays, we needed to confirm that they hadn't lost any important lipid for virulence. For this reason, the strains with a positive neutral red stain were selected to carry on with the experiments. Nontheless, further lipid analysis apart from neutra red stain should be performed to assure the integrity of the cell envelope.

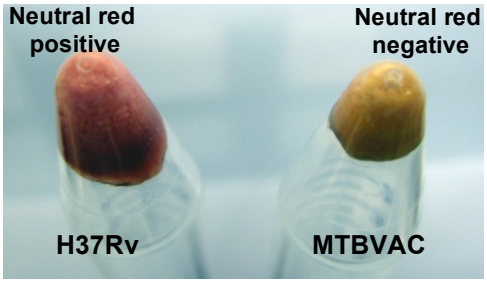


Fig.19. Neutral red stain of H37Rv (positive control) and MTBVAC (negative control) strains.

All the strains had the same lipid profile

By thin layer chromatography, a qualitative lipid analysis, we could answer two important questions: i) if the knock-out strains had lost any free-lipid of the cell wall as a consequence of the deletion of the efflux pump and, ii) if the strains kept the integrity of the cell envelope, which is important for virulence.

First, we extracted free lipids and mycolic acids from the cell envelope of the wild type H37Rv strain and its derivatives (Table 12), as well as GC1237 Beijing strain.

Gene	Knock-out strains	Complemented strains	Overexpression strains
Rv1258c	H37Rv KOTAP	H37Rv KOTAPc	H37Rv pPAZ11
Rv2333c	H37Rv KOSTP	H37Rv KOSTPc	H37Rv pSAN17
Rv1410c	H37Rv KOP55	H37Rv KOP55c	H37Rv pCVZ1

Table 12. Strains used for lipid extraction and TLC analysis.

Several TLC were performed, each one separating and revealing a group of lipids depending of their molecular polarity. All the H37Rv strains showed the same lipid profile, so we can conclude that the deletion of Rv1258c, Rv2333c or Rv1410c has no effect on the composition of the outer lipid layer of the cell envelope. However, it should be noted that this is a general qualitative analysis, so there could be differences only detected with more specific techniques.

H37Rv is not producing a functional Pks3

Pks3 is a polyketide synthase involved in the synthesis of mycolipanolic and mycolipenic acids, the trimethyl branched fatty acids present in the polyacyltrehalose of *M. tuberculosis* (10). In the TLC analysis, we observed that H37Rv wild type strain and its derivatives didn't seem to have polyacyltrehaloses, while the Beijing strain GC1237 did. It has been reported that H37Rv Pasteur, the strain sequenced by *Cole et al.*(27), has a C->A mutation at position 1467 of the *pks3* gene, resulting in a stop codon, whereas other strains such us CDC1551 and ATCC 2729 have a tyrosine codon (32). In order to find if the H37Rv strain used in our work was not producing a functional Pks3 due to this mutation, this region of both H37Rv and GC1237 was sequenced with oligonucleotides pks3-Fw and pks3-Rv. Consistenly with the TLC results, we found that H37Rv did have this mutation, and GC1237 didn't.

H37Rv and the knock out mutants have similar growth rates in liquid medium

The deletion of an efflux pump can affect the fitness of bacteria. With the aim of testing the growth rate of the efflux pumps knock out mutants, OD_{600nm} of H37Rv wild type, H37Rv KOTAP, H37Rv KOSTP and H37Rv KOP55 were measured for a month. When the cultures reached OD_{600nm}~0.5, they were used to start another growth rate experiment. A total of three consecutive experiments were done, and this was performed in duplicate.

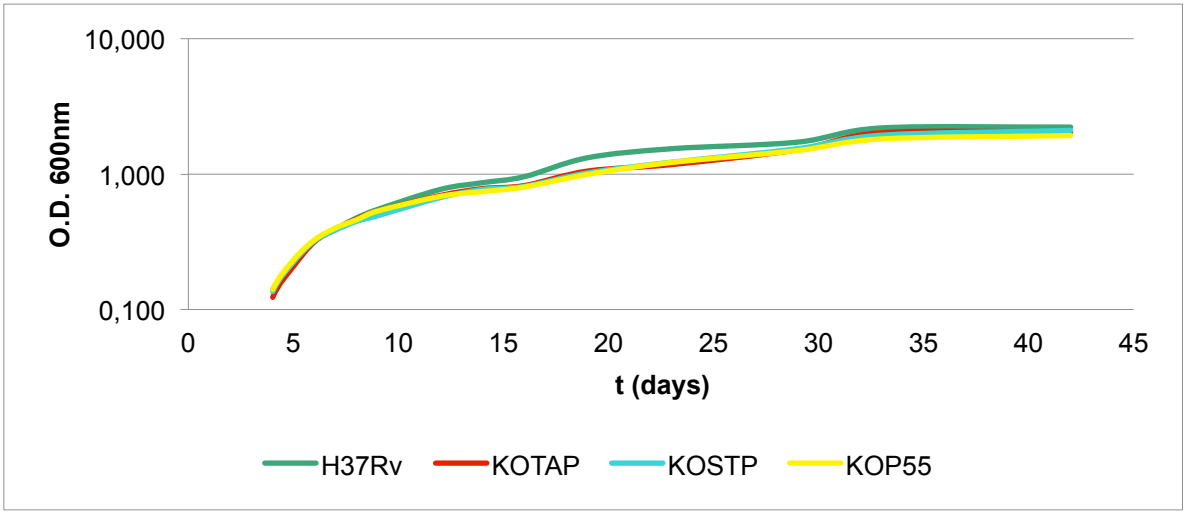


Fig. 20. Growth curves of H37Rv, H37Rv KOTAP, H37Rv KOSTP and H37Rv KOP55.

As it is observed in Fig. 20, no significant differences were noted between the wild type and the knock-out strains. However, a possible difference could be masked by the nutrient-rich media; if these efflux pumps are transporting any molecule important for the fitness of bacteria, maybe it can only be visualized in a minimum medium.

The P55 knock-out mutant and overexpressing strains have limited growth ability on solid media

Both knock-out and overexpression strains formed smaller colonies than the wild-type after two weeks of incubation. However, the colony of overexpression strain was rougher than the one of knock out mutant.

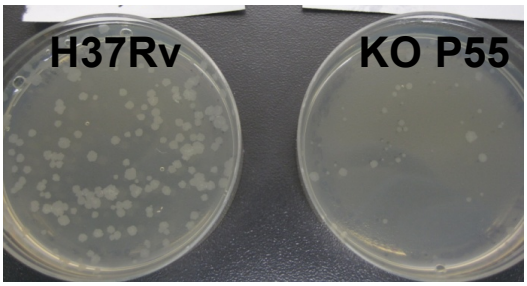


Fig 21. Comparison between morphologies of wild type (H37Rv) and P55 knock-out (KO P55) colonies.

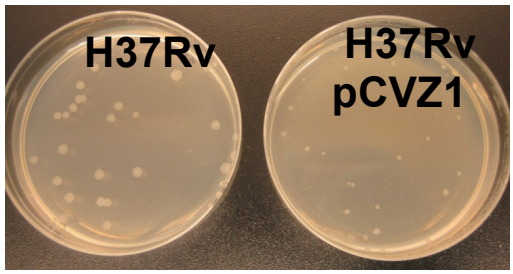
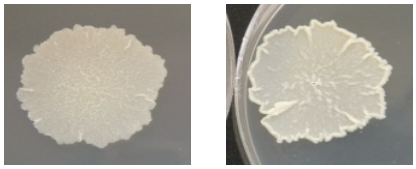


Fig 22. Comparison between morphologies of wild type (H37Rv) and P55 overexpression

Deletion of P55 efflux pump results in a flat colony, and the rough texture is restored in the complemented strain.



KOP55 KOP55c

Fig 23 Morphology of P55 knock-out mutant (KOP55) and complemented strain (KOP55c) colonies after two months of growth.

Susceptibility assays

Minimum Inhibitory Concentrations were calculated by the resazurin method. Results of susceptibility assays are showed in Tables 13-15. Differences in susceptibility are shaded.

Increased susceptibility to spectinomycin (16-fold), gentamicin (2-fold) and streptomycin (2-fold) was found for *Rv1258c* knockout mutant. Deletion of *Rv1410c* caused a decrease in MICs of vancomycin and rifampicin (8-fold). Accordingly, the complemented knockout mutants showed wild type susceptibility levels. Rv2333c knockout mutant showed increased susceptibility to vancomycin, but wild type susceptibility levels are not reached in the complemented strain.

Rv1258c			
Compound	**MIC (mg/l)**		
	H37Rv	**KOTAP**	**KOTAPc**
Spectinomycin	128-64	8-4	64-32
Streptomycin	0,4	0,2	0,4
Ethambutol	2	2	2
Gentamicin	4	2	4
Isoniazid	0,4	0,4	0,4
Tetracyclin	4	4	4
Triclosan	32	32	32
Chloramphenicol	4-2	4-2	4-2
Acriflavine	2	2	2

Table 13. MICs of *Rv1258c* knock out and complemented strains.

Rv1410c			
Compound	MIC (mg/l)		
	H37Rv	KOP55	KOP55c
Isoniazid	0,4	0,4	0,4
Gentamicin	4	4	4
Triclosan	32	32	32
Vancomycin	4-2	0,5-0,25	2-1
Rifampicin	0,0128-0,0064	0,0016	0,0064
Tetracyclin	4	4	4
Chloramphenicol	4-2	4-2	4-2
Acriflavine	4	2	2

Table 14. MICs of *Rv1410c* knock out and complemented strains.

Rv2333c			
Compound	MIC (mg/l)		
	H37Rv	KOSTP	KOSTPc
Rifampicin	0,0064	0,0064-0,0032	0,064-0,0032
Chloramphenicol	4	4	4
Triclosan	32	32	32
vancomycin	4-2	1	1
Isoniazid	0,4	0,4	0,4

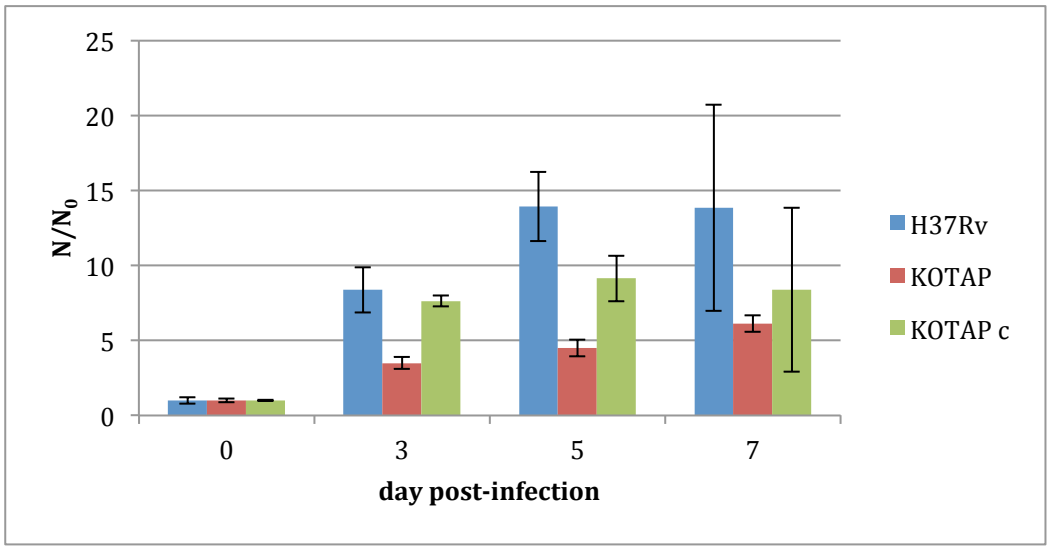
Table 15. MICs of *Rv1410c* knock out and complemented strains

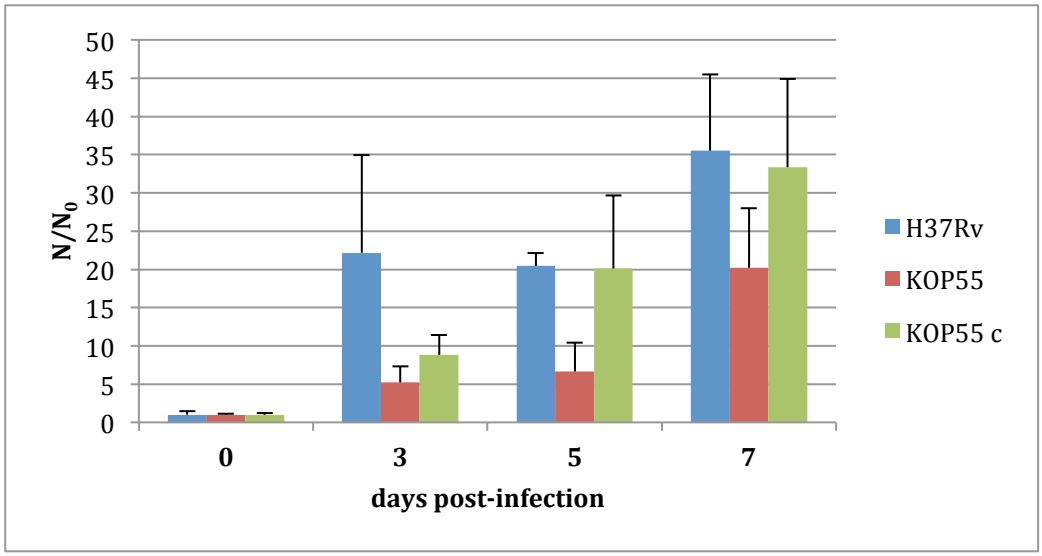
Infection assays

Intracellular replication in J774

Intracellular replication of knock-out, complemented and the wild type strains was tested in J774 mouse macrophage-like cells. The multiplicity of infection (MOI) was 0.5-1 bacteria per macrophage. At 4 h and 1, 3, 5 and 7 days post-infection, intracellular bacteria were counted. (Fig 23, 24, 25).

Infection assays in mouse macrophages showed that Rv1258c, Rv2333c and Rv1410c efflux pumps could be implicated in virulence, as they have less intracellular replication than the wild type.





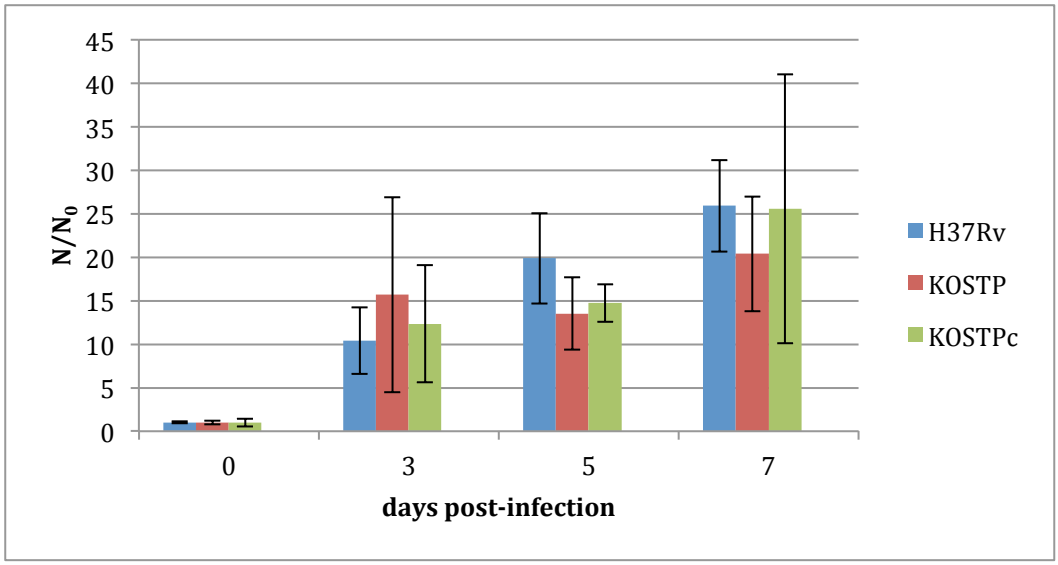


Fig. 23. Replication of efflux pump mutants referred to 4h post-infection in J774 mouse macrophage-like cells. **N**: number of viable intracellular bacteria on the respective day post- infection. **N_0**: number of viable intracellular bacteria on 4h post- infection. **A**. Rv1258c mutants. **B**. Rv2333c mutants. **C**. Rv1410c mutants.

Apoptosis induction in cells J774

Level of apoptosis induced by wild-type and knock-out mutants was determined in J774 mouse macrophage cells. J774 cells were infected with H37Rv, KOTAP and KOP55 and marked with annexin-V and 7-AAD. By analysis of J774 infected cells by flow citometry, the proportion of cells in an apoptotic state can be found. The apoptotic cells show annexin fluorescence, but don't show 7-AAD fluorescence.

No differences in the proportion of apoptotic cells infected with wild-type and knock-out mutants were found (Fig 24).

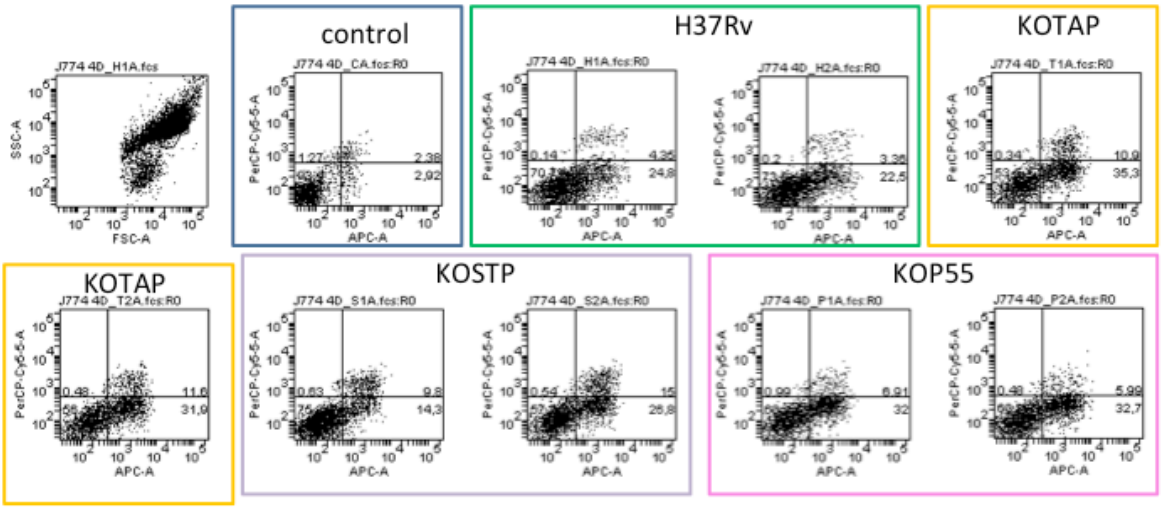


Fig.24. Analysis of annexin-V and 7-AAD fluorescence of J774 cells by flow citometry.

P55 shows an attenuated phenotype in mouse model infection

The wild type strain and the three knock-out mutants were tested in immunocompetent mice C57BL/6. Infection was carried out inoculating 150-300 CFUs via intratracheal injection. After three weeks of infection, mice were sacrified and viable counts were performed on serial dilutions of the homogenized lungs. Table x shows the Replication of efflux pumps mutants in mouse lung.

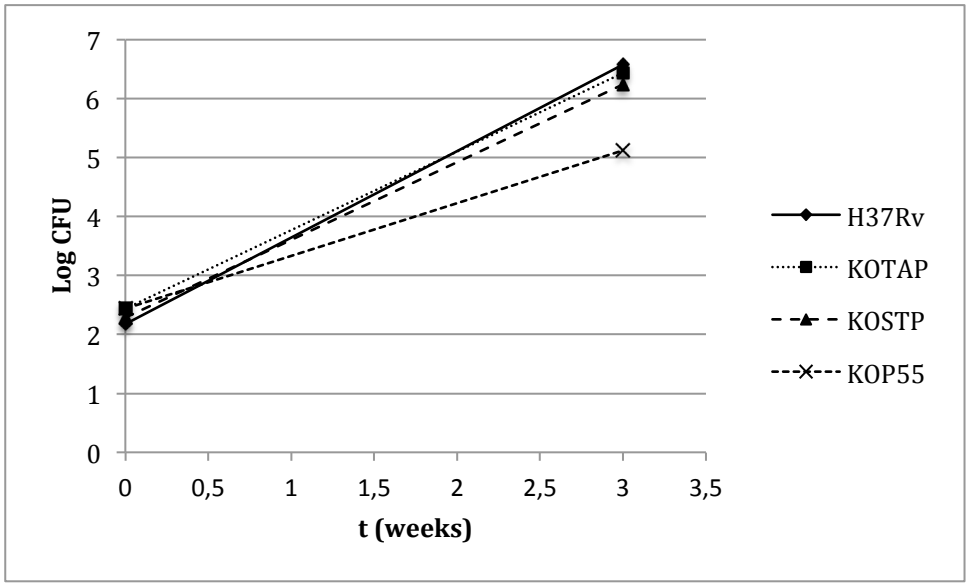


Fig. 25. Replication of efflux pumps mutants in mouse lung. Average Log CFU of the inoculum suspension and at week 3 post-infection.

Among the three knock out mutants, the only one that showed an attenuated phenotype in immunocompetent mice was KOP55. However, these experiments are going to be done in immunodeficient mice, in which we can see more clearly if there is a contribution to virulence.

Conclusions

- The efflux pump Rv1258c (Tap) of *M. tuberculosis* is implicated in intrinsic resistance to streptomycin, gentamicin and spectinomycin. The knock out mutant of *tap* didn't show a difference in susceptibility to tetraclycine compared to the wild type H37Rv strain, as it did with *M. bovis* BCG model. Infection assays in mouse macrophage cell model revealed that Tap could have a role in virulence. However, the knock-out mutant of *Rv1258c* had the same phenotype as H37Rv in infection assays in immunocompetent mouse model.

- Rv1410c (P55) efflux pump of *M. tuberculosis* is contributing to intrinsic resistance to rifampicin, one of the two main antibiotics in antituberculous treatment, and vancomycin. H37Rv knock-out mutant of Rv1410c has altered growth in solid media: it grew more slowly than the wild-type and showed a flatter and smoother morphology of colony. Mouse macrophage cell infection assays and in vivo infection (immunocompetente mice) showed that P55 has a role in virulence of *M. tuberculosis*.

- H37Rv knock-out mutant of Rv2333c *(*Stp*)* didn't show increased susceptibility to tetracycline and spectinomycin, as it did *M. bovis* BCG *Rv2333c* knock-out mutant. Infection assays in mouse macrophage cell model revealed that Stp could have a role in virulence. However, infection assays of immunocompetent mice showed no difference between the replication in lungs of the knock-out mutant of *Rv2333c* and that of H37Rv wild-type strain.

- Rv1258c, Rv2333c and Rv1410c don't seem to be implicated in apoptosis induction of mouse macrophage cells.

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Chapter 2

Analysis of mutations conferring streptomycin resistance of *M. tuberculosis* reveals a SNP in *Rv1258c* gene

This part of the study is presented as a manuscript, which was submitted and is currently under review, with positive feedback from the referees, and high possibility of being published.

Analysis of mutations in streptomycin resistant strains reveals a simple and reliable genetic marker for identification of *Mycobacterium tuberculosis* Beijing genotype

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Keywords: tuberculosis, genotyping, Beijing family, efflux pump, streptomycin.

Running title: SNP in *M. tuberculosis* Beijing lineage

ABSTRACT

Mycobacterium tuberculosis pandemic is a major health problem worldwide, further complicated by increasing incidence of drug resistant isolates and the existence of highly transmissible strains, such as those of the Beijing family. We have analysed streptomycin (STR) resistant *M. tuberculosis* clinical isolates, looking for mutations in *rpsL*, *rrs* and *gidB* genes. In addition, we also sequenced Rv1258c gene encoding Tap, an efflux pump transporting STR. Mutations affecting codons 43 and 88 of *rpsL* gene were found in 44,4% of the strains, and 16,7% of the strains carried mutations in *rrs* gene, probably contributing to STR resistance. Many strains presented mutations in *gidB* gene but their implication in STR resistance remains unclear. Interestingly, we found a cytosine nucleotide insertion between positions 580 and 581 of the *Rv1258c* gene (denominated Tap^{580}) in all Beijing isolates included in this study, suggesting it could be a novel polymorphism specific of the Beijing family of *M. tuberculosis*. We have developed a simple and fast PCR-RFLP method for detecting Tap^{580} insertion, that was applied to screen a collection of 220 DNA samples obtained from cultures of *M. tuberculosis* isolates and seven respiratory specimens. In all cases, we were able to identify correctly all the Beijing and non-Beijing representatives. Tap^{580} is a novel polymorphism specific of the highly transmissible Beijing family, which allows a fast detection of these strains even at very early stages of infection.

INTRODUCTION

Mycobacterium tuberculosis still constitutes the cause of millions of incident cases of tuberculosis and deaths worldwide (28), many of them among HIV-positive patients. This global pandemic is further complicated by the increased incidence of drug resistant strains (28), which generally have accumulated mutations in genes encoding drug target or drug activating enzymes. Nevertheless, there are resistant strains in which no mutations linked to drug resistance have been identified, hence suggesting yet unidentified drug target proteins or novel mechanisms of drug resistance. A paradigm of this is the case of STR, a first line antituberculosis drug of increasing use in our days due to the raising incidence of multi and extensively drug resistant strains (MDR and XDR respectively). Mutations in genes *rpsL*, *rrs* and *gidB* (encoding S12 ribosomal protein, 16S rRNA and ribosome methyltransferase, respectively) have been found in ca. 70% of *M. tuberculosis* isolates resistant to STR; in the rest of STR resistant *M. tuberculosis* strains, the cause of drug resistance remains unknown.

Molecular techniques have allowed the differentiation of several lineages and families among *M. tuberculosis* strains, which differ not only in global geographical distribution but also in terms of transmissibility, drug resistance, treatment outcome and other factors. The Beijing family of *M. tuberculosis* isolates are characterised by their increased ability to spread and cause disease and their increased association with drug resistance (10). Because of this, many methods have been developed aimed at identifying and differentiating *M. tuberculosis* Beijing isolates (4), (16), (20), (14). There are several methods for identifying Beijing isolates, including spoligotyping, RFLP using IS*6110* as a probe, or the presence of a copy of IS*6110* in the *dnaA* region (12); others are based on the detection of specific genomic deletions such as RD105 or an intact *pks15/1* gene (24). Recently, the detection of a single-

nucleotide polymorphism (SNP) in the *Rv2629* gene by using real-time PCR followed by high-resolution melting has also been described (4).

In this work, we present the analysis of target mutations in clinical isolates of *M. tuberculosis* resistant to STR. In addition, we investigated the nucleotide sequence of *Rv1258c* gene encoding the drug efflux pump Tap from *M. tuberculosis*, which transports STR and other antibiotics, as we have previously described (2), (8), (19), as a potential STR resistance determinant. We identified a nucleotide insertion in the coding sequence of the *Rv1258c* gene, which does not seem to be associated with resistance to this drug. Interestingly, this insertion is exclusive of the Beijing family of *M. tuberculosis* isolates and has led to the development of a method for screening *M. tuberculosis* strains (either purified DNA or directly on respiratory samples) and reliably identifying those belonging to the Beijing family of strains.

MATERIALS & METHODS

M. tuberculosis DNA samples

First, DNA samples of 18 *M. tuberculosis* clinical isolates resistant to STR and some additional drugs (including several MDR isolates), of the Universidad de Zaragoza culture collection, along with the control strain H37Rv (fully drug susceptible) were used as template to amplify and sequence the target genes (*rpsL*, *rrs*, *gidB* and *Rv1258c*). This group included 4 strains that had a spoligotyping consistent with being of the Beijing family.

Next, we analysed 220 DNA samples from *M. tuberculosis* complex clinical isolates collected from Hospitals in Huesca, Zaragoza, Madrid and Barcelona (Spain). These isolates included samples from a Spanish national survey on MDR *M. tuberculosis* done between 1998 and 2009. This set included 49 DNA samples having a spoligotyping consistent with those of the Beijing family, 158 were representative of other *M. tuberculosis* distinct lineages (LAM, T, X, S, Haarlem, CAS, EAI and others, including 5 isolates of the MTZ strain, a highly transmissible *M. tuberculosis* strain that has caused major outbreaks in Zaragoza (13) and 13 were *M. africanum* (Table 3). Overall, we got isolates representative of most of *M. tuberculosis* genetic lineages described (9). In these samples, we investigated the presence of the novel nucleotide insertion in *Rv1258c* gene described in this work. We selected 30 DNA samples (18 from Hospital Germans Trías i Pujol, Badalona and 12 from Hospital Gregorio Marañón, Madrid), those originated from STR resistant isolates (five of them belonged to the Beijing family), and the *Rv1258c* gene was completely sequenced in order to verify the presence of the nucleotide insertion in *Rv1258c* gene described in this work, and to identify other potential mutations.

PCR amplification and sequencing

The primers used for amplifying and sequencing genes related with STR resistance, the annealing temperature used in the PCR reaction, and the size of the product are listed in Table 1. PCR reactions were performed in a final volume of 50 µl using 200 µM of each dNTP, 5 µl of Buffer (10x PCR Buffer, Applied Biosystems), 1.25 U of Taq Gold polymerase (Applied Biosystems), 0.25 µM of each primer, 5 µl of dimethyl sulphoxide and 2 µl of DNA (20-100 ng/µl). Amplifications consisted of an initial step at 94 ºC for 10 min, followed by 40 cycles of 1 minute at 94 ºC, annealing for 2 minutes (see Table 1) and 2 minutes at 72 ºC, with a final extension step of 10 min at 72 ºC. All genes were amplified in one single product, except *Rv1258c* gene that was amplified in two overlapping products, one containing the promoter and the first half of the coding sequence, and the second containing the rest of the gene.

PCR products were purified using ExoSAP-IT (Affymetrix) and sequenced using the same primers used for the amplification. Sequences were then analysed by comparison with that of the reference H37Rv strain (Cole *et al*. (6); TubercuList, http://tuberculist.epfl.ch/).

Screening of nucleotide insertion in *Rv1258c* gene

Firstly, an internal fragment of *Rv1258c* gene containing the location of the cytosine nucleotide insertion between positions 580 and 581 of the coding sequence of the *Rv1258c* gene (denominated Tap^{580}) was amplified using primers ctap9 and ctap10 (Table 1). DNA samples were processed 9 minutes at 94 ºC, followed by 35 cycles of 30 seconds at 94 ºC, annealing for 30 seconds at 65 ºC, extension at 72 ºC for 1.5 minutes, and final extension at 72 ºC for 10 minutes. We obtained a product of 1052 bp, which was digested for 1 hour at 37 ºC with *Xho*I restriction enzyme, followed by electrophoresis in 0.8% agarose gel in TBE buffer. As a control of endonuclease digestion, PCR products were digested for 1 hour at 37 ºC with *Pvu*II restriction enzyme, which produces two fragments of 610 and 442 nucleotides for both Beijing and non-Beijing isolates.

Bioinformatic analysis

Databases of bacterial genomes were analysed using the program Blast at NCBI (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/) in search for Tap^{580} insertion in *Rv1258c* gene among sequenced genomes of the *M. tuberculosis* complex. Last access was on July 26th 2012.

Screening of Tap^{580} insertion in *Rv1258c* gene directly in respiratory samples

Seven respiratory samples from patients diagnosed with tuberculosis were obtained from Servicio de Microbiología (Hospital Universitario Lozano Blesa, Zaragoza, Spain). All samples were smear positive (their bacillary load ranged from 2 to >100 bacilli/field) and culture positive. Samples were decontaminated by N-acetyl-L-cysteine/NaOH, and a fraction was incubated 20 minutes at 80 ºC for killing the live bacilli and extracting DNA using the kit GenoLyse® (Hain Lifescience).

RESULTS & DISCUSSION

Mutations in *rpsL*, *rrs* and *gidB* genes

In order to find the mutations responsible for STR resistance in 18 *M. tuberculosis* clinical strains, we sequenced first the genes *rpsL*, *rrs* and *gidB* that are known to carry mutations related with resistance to STR and compared them with the sequence of STR-susceptible H37Rv laboratory strain available in the TubercuList database (http://tuberculist.epfl.ch/). All strains analysed in our study, including the reference H37Rv strain, had mutations A363G in *rpsL* and C299T in *gidB,* which have been reported as sequencing errors in the sequence of H37Rv included in both TubercuList database and GenBank entry AL123456.2 Ioerger, et al. (11), (31).

Significant mutations found in the strains analysed in our study are summarised in Table 2.

Eight of the strains tested (44.4%) carried mutations in *rpsL* gene, being the mutation A128G found in six of them. The four Beijing strains included in this group carried this mutation, which is the most frequent mutation found in STR resistant *M. tuberculosis* Beijing strains (17, 23). Two other strains (11.1%) had the mutation A263G.

Four strains (22.2%) harboured mutations in the *rrs* gene. Three strains carried mutations C340T, A324G and A736G, respectively; to our knowledge this is the first report on mutations in this region. The latter strain also carried mutation C462T according to Tuberculist numbering, mutation that has been already documented as C461T (5). Finally, one strain carried mutation C492T close to the 530 loop of the secondary structure of *M. tuberculosis* 16S rRNA; this strain is further discussed below.

The role of GidB protein in conferring high-level STR resistance in *M. tuberculosis* has been fully characterised (29), although the contribution of certain point mutations to

STR resistance is still controversial; it has been speculated that some mutations in *gidB* gene would promote the acquisitions of mutations in either *rpsL* or *rrs* (18). A large number of mutations (including many silent mutations) were found in the *gidB* gene, in fact, only four strains had a *gidB* gene identical to that of H37Rv. Many missense mutations have been described in STR susceptible strains, such as the mutation T47G (the most frequent mutation found in our study) that has been reported as specific of the Latin-American-Mediterranean (LAM) family strains (21)); in fact, the 6 strains carrying this mutation belonged to the LAM family of strains (data not shown). The four Beijing strains carried two mutations: A276C (E92D) that has been described as a SNP specific of this family (21), and A615G; both mutations can be found in STR susceptible strains (29). In the Beijing strains, these *gidB* mutations occur simultaneously to mutation A128G in *rpsL* gene as mentioned above.

Among the non-Beijing strains, *gidB* C413T mutation has been found both in STR resistant and susceptible *M. tuberculosis* isolates (18, 26, 29). In one STR resistant isolate, we found mutation G248C, likely indicating that it could lead to resistance to this drug; however, other mutations in this position (G248T) have been found in STR susceptible strains (18). Finally, the only *gidB* mutation potentially related with resistance to STR could be the missense mutation G490C, since this one was detected in a strain lacking mutations in *rpsL* and *rrs* genes.

We found mutations in all three genes in one strain; these were *rpsL* A128G, *rrs* C492T and *gidB* T47G. In this strain STR resistance will be due to the mutation *rpsL* A128G, since the two other mutations have been found in both STR resistant and susceptible isolates. Mutation *rrs* C492T, also reported elsewhere as C491T, has been associated to LAM3 genetic lineage of *M. tuberculosis* (25, 27). This very same genetic lineage of *M. tuberculosis* also carries mutation T47G as mentioned above (21).

Finally, one of the STR resistant strains lacked mutations in any of these three genes. In three others, only mutations in *gidB* were found, although these were either silent or have been described also in STR susceptible strains, hence making unlikely that they may have a major contribution to resistance to this drug. This confirms that other mechanisms must contribute to STR resistance in *M. tuberculosis*.

Mutations in *Rv1258c* gene

We hypothesize that since STR is a substrate of Rv1258c efflux pump (19), mutations affecting expression levels of *Rv1258c* gene or changing kinetic properties of the efflux pump could contribute to STR resistance. To further investigate this, we amplified and sequenced the *Rv1258c* gene in the 18 samples of *M. tuberculosis* STR resistant clinical strains in which we had sequenced *rpsL, rrs and gidB* genes. We found two different mutations in *Rv1258c* gene. One strain has a deletion of the adenine nucleotide in position 13 (Table 2) producing a frameshift; as a result, a TGA stop codon will end translation of a peptide of only 10 amino acids. Four strains have an insertion of a cytosine nucleotide between positions 580 and 581 (Table 2); this insertion, which in this work we will refer to as Tap^{580}, causes a frame-shift mutation from codon 194 onwards, resulting in a shorter protein (231 amino acids). This protein probably would not constitute a functional membrane transporter, since it contains only 6 transmembrane segments (TMS) in comparison with the 12 TMS of the full-length protein (419 amino acids) (Figure 1). Bacterial drug efflux pumps of the Major Facilitator Superfamily have 12 or 14 TMS, which are required for transport activity.

These five strains carrying mutations in *Rv1258c* gene also carried mutations A263G (K88R) or A128G (K43R) in *rpsL* gene, which probably will be responsible for the high-level STR resistance making difficult to analyse the contribution of *Rv1258c* mutations in resistance to this drug.

Remarkably, we noticed that the four strains carrying Tap^{580} insertion had been typed as belonging to the Beijing lineage by either RFLP and/or spoligotyping (data not shown). We hypothesized that this could represent a novel polymorphism specific of the Beijing family. To test this, we inspected the presence of Tap^{580} insertion in thirteen genomes of *M. tuberculosis* available in public databases (NCBI, last access was on July 26th 2012), including clinical isolates, laboratory strains such as H37Rv and H37Ra, and two strains of the Beijing family. Interestingly, only CCDC5079 and CCDC5081 that belong to Beijing family (30) had the Tap^{580} insertion (Table 4), further supporting that Tap^{580} is a specific insertion of the Beijing family isolates. Other species of the *M. tuberculosis* complex, such as four substrains of *M. bovis* BCG and *M. bovis* AF2122/97 did not show Tap^{580} polymorphism and had complete identity with *Rv1258c* gene of H37Rv (Table 4).

The Tap^{580} insertion is present in clinical strains of the Beijing family of isolates

We further investigated the presence of Tap^{580} insertion in different subtypes of the Beijing family of isolates, and in isolates of other genetic families.

For this, we first developed a quick and simple method for detecting Tap^{580} insertion. Between positions 577 and 582 of the *Rv1258c* gene, the sequence CTCGAG is the target for *XhoI* endonuclease; insertion of a cytosine nucleotide in Beijing strains results in CTCGCAG that is not recognized by this endonuclease (Figure 2). We designed two primers for amplifying a fragment of 1052 bp of the *Rv1258c* gene, from nucleotides 165 to 1216, which includes the position of the Tap^{580} insertion. Digestion of PCR products with *XhoI* endonuclease resulted in two DNA fragments of 413 and 639 bp in the case of non-Beijing strains, whereas PCR products from Beijing strains remained unaffected (Figure 2).

We proceeded then to screen a collection of 220 clinical isolates of *M. tuberculosis* complex, which included 49 isolates having spoligotyping consistent with that of the

Beijing family. DNA samples were given random numbers and were blind tested using our PCR-RFLP method described above. We found that Tap^{580} insertion was present in all Beijing strains and absent in all strains belonging to other lineages of *M. tuberculosis*; Tap^{580} insertion was also absent from *M. africanum* isolates (Table 3).

We included two controls in our assays. First, in each experiment we included samples of DNA from the laboratory strain *M. tuberculosis* H37Rv (which does not belong to the Beijing family of isolates), and from the *M. tuberculosis* GC1237 strain (a Beijing isolate recently characterised (3)). The PCR product from *M. tuberculosis* H37Rv was cut by *Xho*I, whereas that of *M. tuberculosis* GC1237 strain was not cut, confirming that all steps in the identification process were carried out satisfactorily. Second, all PCR products were digested with *Pvu*II restriction enzyme, which cuts the amplification products of Rv1258c gene obtained from both Beijing and non-Beijing samples, demonstrating that the amplification step has been specific of Rv1258c gene and ruling out the possibility of unspecific amplifications.

Finally, out of the 220 samples, we sequenced *Rv1258c* gene in the 30 *M. tuberculosis* STR resistant clinical isolates from Hospital Gregorio Marañón (Madrid, Spain) and Hospital Germans Trias i Pujol (Badalona, Spain). We found consistently that Tap^{580} insertion was present in the 5 Beijing and absent in all the non-Beijing strains, hence validating the method for screening Tap^{580} insertion developed in this work. In addition, we found a deletion of 4 nucleotides spanning positions 835 and 838 of the *Rv1258c* gene in one of the Beijing samples, which is located downstream of the in-frame stop codon in position 694-696 produced by Tap^{580} insertion.

Detection of the *Rv1258c* nucleotide insertion in clinical samples

In order to assess the usefulness of this technique for detecting Beijing strains directly in clinical samples, we analysed seven respiratory samples with diverse bacillary load and positive for culture of *M. tuberculosis*. After isolating the DNA

from sputum samples, we successfully amplified the 1052 bp PCR product from all samples, including those having a low bacillary load, and digested with *Xho*I endonuclease. All seven *M. tuberculosis* strains present in sputum samples were identified as non-Beijing, in agreement with spoligotyping.

The precise effect of this nucleotide insertion in the physiology of *M. tuberculosis* Beijing isolates remains to be fully elucidated. Recently, the role of Rv1258c efflux pump in drug tolerance in *M. tuberculosis* has been reported (1). Since the identified single nucleotide insertion in *Rv1258c* gene in Beijing isolates results in a truncated, and most probably inactive protein, this would suggest that isolates carrying this nucleotide insertion could have a disadvantage in terms of drug resistance and drug tolerance in comparison with others carrying a fully functional Rv1258c transporter. However, since Beijing isolates are frequently associated with a higher propensity to acquire drug resistance, it is conceivable that other mutations are actually compensating the loss of Rv1258c in this family of *M. tuberculosis* isolates. Examples of compensatory mutations occurring following the acquisition of drug resistance associated mutations have been described for *M. tuberculosis* (7).

In summary, we have found a new genetic polymorphism of *M. tuberculosis* Beijing strains, which has been used to develop a simple and reliable technique for identifying isolates of this family. Given the higher transmissibility rate of this family of strains, which in addition are more prone to develop drug resistance, methods for rapid identification constitute a very important tool for the control of outbreaks caused by isolates of the Beijing family of strains. This technique is fast since it can be performed directly on clinical specimens and there is no need for culturing strains, making it an ideal method for being implemented in those settings where routine culture of clinical samples cannot be done. In addition, this technique is easy to carry out and does not require sophisticated equipment since only a thermocycler and DNA electrophoresis system are needed. In addition, samples can be processed

individually or in groups of any size, and this can easily accommodate to the workflow of a clinical laboratory, without the need for processing samples in groups of a fixed number. All this, along with the specificity for both detection of *M. tuberculosis* and identification of Beijing family of strains, make it ideal for implementation in clinical laboratories, specially in those settings with a high incidence of tuberculosis infections caused by this family of strains.

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FIGURES

Figure 1. Hydrophobicity profile and transmembrane prediction of the Rv1258c proteins of *M. tuberculosis* H37Rv (A) and Beijing isolates (B), using the Hidden Markov Model (TMHMM Server v. 2.0, Center for Biological Sequence Analysis, Technical University of Denmark, http://www.cbs.dtu.dk/services/TMHMM/).

Figure 2. Screening of the insertion Tap^{580} of Beijing strains. A. DNA gel electrophoresis showing PCR product with primers ctap9 and ctap10 digested with *Xho*I. M. Size marker, lambda DNA digested with *Pst*I. 1. GC1237 (Beijing). 2. H37Rv (non Beijing). B. Sequence of the region of Rv1258c gene in a Beijing strain (1) and H37Rv strain (2) (GenBank AL123456.2).

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TABLE 1. Primers used for amplification in this study.

Target	PCR product (bp)	Ta[c] (ºC)	Primer	Sequence 5'->3'	Reference
For amplifying and sequencing					
rpsL	501	52	S13	GGCCGACAAACAGAACGT	(15)
			S16	GTTCACCAACTGGGTGAC	(15)
rrs	1037	58	264	TGCACACAGGCCACAAGGGA	(22)
			285	GAGAGTTTGATCCTGGCTCAG	(22)
gidB	977	56	gidB3	GAACGGAAGATCGTCCAC	This work
			gidB4	CGATAGTTGAAGCCTGGC	This work
Rv1258c[a]	830	58	ctap1	CAATGTGGATTACCGCGAC	This work
			ctap2	GTCTTGCCGGTAGCCGTC	This work
Rv1258c[b]	902	58	ctap3	CGCAGGTTCCAGACGAAG	This work
			ctap4	GATCAGCGCGTTGAGTTC	This work
For detection of nucleotide insertion					
Rv1258c	1052	65	ctap9	GGCCGACAAACAGAACGT	This work
			ctap10	GTTCACCAACTGGGTGAC	This work

[a] 3' region of *Rv1258c* gene.

[b] Promoter and 5' region of *Rv1258c* gene.

[c] Ta= annealing temperature

TABLE 2. Mutations found *in rpsL, rrs, gidB* and *Rv1258c* genes in strains of the Universidad de Zaragoza culture collection.

M. tuberculosis SM-resistant	Mutation found in:						
Strain	*rps*L		*rrs*	*gidB*		*Rv1258c*	
	DNA	Protein	DNA	DNA	Protein	DNA	Protein
Z-07044			C340T				
Z-07047[a]	A128G	K43R		A276C[b]	E92D	InsC 580-581	194 frameshift
				A615G[b]	silent		
HMS-1838				C413T[b]	A138V		
HCU-2879	A263G	K88R					
HCU-2934	A128G	K43R		T47G[b]	L16R		
HCU-2830[a]	A128G	K43R		A276C[b]	E92D	InsC 580-581	194 frameshift
				A615G[b]	silent		
HMS-1695	A128G	K43R	C492T	T47G[b]	L16R		
HMS-1691	A263G	K88R				A13del	5 frameshift
HMS-1781				G490C	G164R		
VEN-4145[a]	A128G	K43R		A276C[b]	E92D	InsC 580-581	194 frameshift
				A615G[b]	silent		
VEN-1714				T47G[b]	L16R		
				C409G[b]	R137G		

VEN-5292			A324G	T47G[b]	L16R		
				T149C	L50P		
VEN-2457				T47G[b]	L16R		
VEN-1667				T47G[b]	L16R		
				G248C	R83P		
VEN-2543							
VEN-314				C159T	silent		
VEN-4237			C462T	C159T	silent		
			A736G				
VEN-3748[a]	A128G	K43R		A276C[b]	E92D	InsC 580-581	194 frameshift
				A615G[b]	silent		

[a] Strains belonging to Beijing family.

[b] Mutations found in susceptible strains (18, 26, 29)

TABLE 3. Number of strains belonging to different lineages used in Tap^{580} screening.

Lineage / species	No. of strains
Beijing	49
T	27
LAM[a]	25
HAARLEM	18
U	16
CAS[b]	12
EAI[c]	9
S	2
X	1
Not assigned	48
M. africanum	13
TOTAL	**220**

[a] LAM: Latin American and Mediterranean lineage

[b] CAS: Central-Asian lineage

[c] EAI: East African-Indian lineage

TABLE 4. Search for Tap^{580} insertion by bioinformatics analysis of *M. tuberculosis* complex sequenced genomes.

Strains	Beijing lineage	Tap ^{580}
Mycobacterium tuberculosis		
H37Ra	No	No
H37Rv	No	No
CCDC5079	Yes	Yes
CCDC5180	Yes	Yes
CDC1551	No	No
CTRI-2	No	No
F11	No	No
KZN 1435	No	No
KZN 4207	No	No
RGTB327	No	No
RGTB423	No	No
str. Erdman = ATCC 35801 DNA.	No	No
UT205	No	No
Mycobacterium bovis		
BCG Pasteur 1173P2.		No
BCG Mexico.		No
BCG. Moreau RDJ		No
BCG. Tokyo 172 DNA.		No
AF2122/97		No

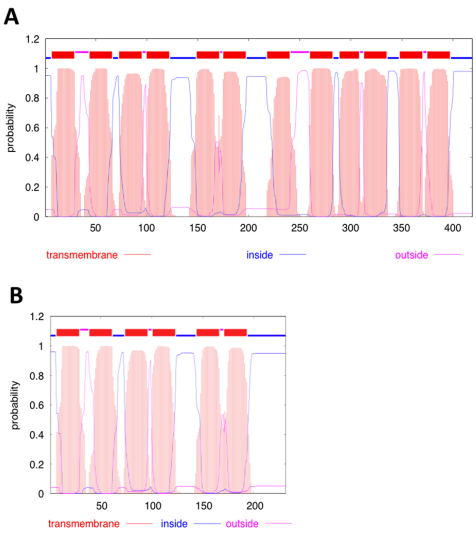

Figure 1. Hydrophobicity profile and transmembrane prediction on the Rv1258c proteins of *M. tuberculosis* H37Rv (A) and Beijing isolates (B), using the Hidden Markov Model (TMHMM Server v. 2.0, Center for Biological Sequence Analysis, Technical University of Denmark, http://www.cbs.dtu.dk/services/TMHMM/).

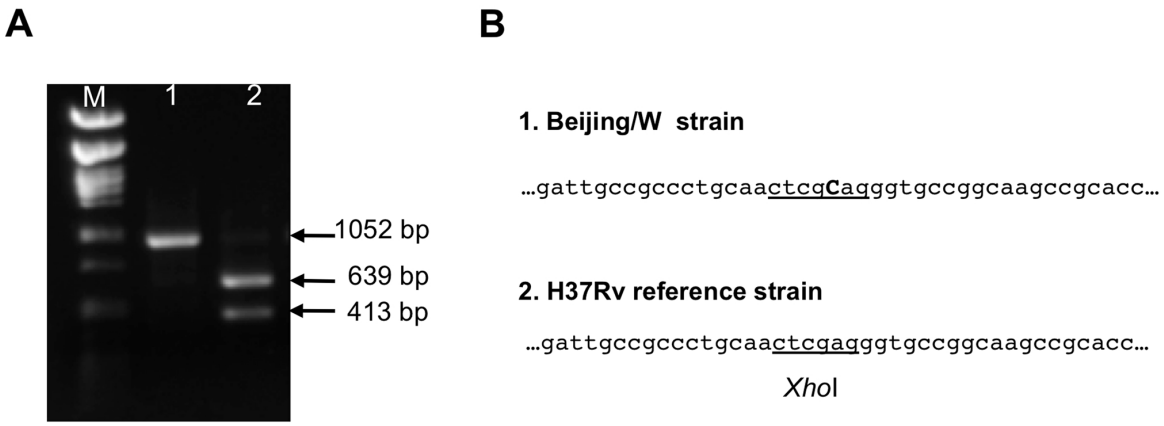

Fig. 2. Screening of the insertion Tap^{580} of Beijing strains.

A. DNA gel electrophoresis showing PCR product with primers *ctap9* and *ctap10* digested with *Xho*I. M. Size marker, lambda DNA digested with *Pst*I. **1**. GC1237 (Beijing). **2**. H37Rv (non Beijing).
B. Sequence of the region of *Rv1258c* gene in a Beijing strain (1) and H37Rv strain (2) (GenBank AL123456.2)

Comments

The Beijing family

The *M. tuberculosis* Beijing genotype family constitutes a homogeneous group of strains sharing a closely related IS6110 RFLP patterns containing a high number of bands, identical spoligotyping (deletion of spacers 1 to 34 in the Direct Repeat region) (106), an insertion of IS6110 between *dnaA-dnaN* genes (54) and one or two IS6110 copies in a DNA region called NTF locus (53, 54)

Different studies have indicated that one-third of global TB cases is caused by Beijing family strains assigning this lineage to one of the most successful mycobacterial families in terms of morbidity and mortality (12). In some areas, such as Vietnam, Cuba, and Estonia, Beijing strains were found to be strongly associated with drug resistance (8, 14, 31, 52). *M. tuberculosis* GC1237, which belongs to the Beijing family, has been responsible for different epidemic outbreaks in the Gran Canary Island since 1991, representing the 25% of the cases of tuberculosis in the year 2007-2008 (24, 64). GC1237 is the strain used for Beijing control in the PCR-RFLP method.

A number of selective advantages have been associated with its success including a lower efficacy of BCG for Beijing strains (60, 106) higher virulence (60, 62), ability to induce a differential immune response (33, 61), higher transmissibility (12) and enhanced capacity to grow in human macrophages and monocytes (57, 112) and an enhanced capacity to acquire drug resistance (2, 13, 14)

The PCR-RFLP method described in the manuscript, constitutes a fast, easy and inexpensive method of detecting Beijing strains, even at early stages of infection. This tool can be helpful to detect this highly virulent and transmissible strains, allowing a better control of outbreaks.

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Chapter 3

Complementation of *E. coli* efflux pumps knock out mutants with MFS and RND transporters of *M. tuberculosis*

This part of the study was done in the "Infectious Diseases Division", University Hospital of Freiburg, Germany.

Introduction

Efflux pumps from RND family

Efflux pumps are present both in gram positive and negative bacteria, and they are related with multidrug resistance and other bacterial processes such us colonization and persistence in the host (78). Major Facilitator Superfamily (MFS) and Resistance-Nodulation-Cell Division Superfamily (RND) are two of the main membrane transporters in bacteria (58).

AcrAB-TolC, the main RND efflux pump in *E. coli*

Escherichia coli has seven RND efflux pumps, from which the most important in multidrug resistance is AcrAB-TolC (19). This transporter is constituted by three assembled proteins: AcrB (the RND protein, located in the inner membrane), AcrA (a periplasmic fusion protein) and TolC (a cannel located in the outer membrane), substrates can be extruded directly to the exterior of the bacterium. Multidrug resistance in *E. coli* is usually asociated with AcrAB-TolC overexpression (77). Other important efflux pumps in *E. coli* are MdfA (MFS) and EmrE (SMR), located in the inner membrane and transporting substrates to the periplasmic space; thus they need to be coordinated with other efflux pumps, such as AcrAB-TolC, to completely export compounds. Ethidium bromide and acriflavine are substrates of both MdfA and EmrE so, if one of them is deleted, the other can compensate the effect. Consequently, if both are deleted, then the effect is similar to the inactivation of *acrB* gene (97).

MmpL efflux pumps, the RND proteins of *M. tuberculosis*.

Mycobacterium tuberculosis contains 13 RND transporters, named MmpL (Mycobacterial Membrane Protein Large), and that have certain homology with AcrB from *E. coli*. Colocalization of mmpL genes with genes of the lipid metabolism induced to think that they may be related with lipid transport. That is the case of MmpL7, which transports pthiocerol dimycocerosate (PDIM) and MmpL8, whose substrate is a SL-1 precursor, both lipids of the mycobacterial cell wall. Part of MmpL proteins are implicated in virulence of *M. tuberculosis*, but no significant contribution to intrinsic resistance has been found (32).

Expression of heterologous genes in *E. coli*

Previous studies have proved that efflux pumps from heterologous microorganisms can be functionally expressed in *E. coli*: MexB from *Pseudomonas aeruginosa* was able to partially complement AcrB knock-out strain of *E. coli* (109), and Mmr from *M. tuberculosis* (a SMR protein) was functional when expressed in *E. coli* (70).

Influence of the codon usage

There are marked differences in codon usage from one organism to another. Significant variation in codon usage patterns among genes in one organism appears to be associated with their expression levels. Genes with a high proportion of optimal codons are highly expressed, whereas those with rare codons are poorly expressed. Moreover, the presence of rare codons can cause ribosome stalling, slow translation, premature translation termination and translation errors and therefore inhibit proper protein synthesis and even cell growth. To avoid the potential expression problems resulting from rare codons, one can either optimize codon usage in the target gene by silent mutations for expression in *E. coli* or expand the intracellular tRNA pool of rare codons by introducing a plasmid which encodes these tRNAs. Both strategies have been successfully used to enhance the expression of heterologous genes with rare codons in *E. coli*. However, negative results have been reported indicating that factors other than codon usage can affect protein expression (43).

Objectives

1. To express Rv1258c and Rv1410c (MFS) efflux pumps of *M. tuberculosis* in *E. coli* wild type, mdfA (MFS) and emrE (SMR) single and double knock out mutants to determine susceptibility assays to different substrates, including those already confirmed in mycobacteria.

2. To express MmpL7 (RND) efflux pump of *M. tuberculosis* in *E. coli ΔacrB*, in order to test its ability to reconstitute a functional AcrA-MmpL7-TolC efflux transporter.

3. To perform efflux and accumulation techniques to corroborate potential substrates of these transporters. This would provide a new and fast model for studying efflux kinetics of mycobacterial efflux pumps using *E. coli*.

4. To characterize *M. tuberculosis* mutants deleted in genes encoding MmpL7 and MmpL10, by determination of their levels of intrinsic drug resistance and other potential physiological alterations.

Materials and Methods

Bacterial strains, media and growth conditions

The strains *Escherichia coli* 3AG100 and BW25113 and derivatives were used in this study (Table 22).

E. coli strains were cultured in Luria-Bertani (LB) broth or on LB agar plates at 37ºC. Antibiotics were added when necessary, at final concentrations listed in Table 16. Liquid cultures were grown overnight in 14ml plastic tubes or erlenmeyer flasks. For *E. coli* strains carrying plasmids derived from pET6AP, IPTG was added in order to induce gene expression.

In this work, we have used *M. tuberculosis* CDC1551 and derivatives (Table 23). All manipulations of *M. tuberculosis* were done in a Biosafety Level 3 Laboratory (BSL3).

Liquid medium for *M. tuberculosis* was Middlebrook 7H9 medium supplemented with 10% OADC (0.05% oleic acid, 0.5% bovine serum albumin, 0.2% dextrose, 0.085% NaCl, 0.0003% beef catalase) (Difco) (7H9/OADC from now). In order to keep the culture clump-free, Tween 80 was added to a final concentration of 0.05%, except for the media used in susceptibility assays, which were supplemented with glycerol 0.5%.

Solid medium was Middlebrook 7H10 or 7H11 medium supplemented with 10% OADC (Difco) (7H10/OADC from now).

M. tuberculosis H37Rv was grown at 37ºc; liquid cultures were done in cell culture flasks, without shaking.

Storage of strains was done in cryovials (MAST CRYOBANK^{TM}). To prepare them, 2-3 colonies were taken from the agar plate and dispersed in the cryogenic preservative solution containing chemically treated beads. After 15 minutes of incubation at room temperature, the solution was removed and the vial containing the beads kept at -80ºC.

Compound	*E. coli*	*M. tuberculosis*
Kanamycin (Km)	20 µg/ml	20 µg/ml
Ampicilin (Ap)	100 µg/ml	-
Tetracyclin (Tet)	3 µg/ml	-
Streptomycin (Sm)	50 µg/lm	-
IPTG	1mM	-

Table 16. Antibiotics and compounds used in this work.

General techniques of nucleic acids

Oligonucleotides

All the oligonucleotides are listed in Tables 17 and 18. Artificial endonuclease restriction sites were added when required and are shown underlined.

Oligonucleotides with cloning purposes		
Oligonucleotide	**Sequence 5'->3'**	**Description**
pET30-ORF6-Fw	GAAGGAGATATACATATGAAAATCGAAG	Sequencing pET6Ap derivatives
MmpL7-Rv	CAGCGTCCAAGAAAATACCG	Sequencing pCVF7
ctap3	CGCAGGTTCCAGACGAAG	Sequencing pCVF1
pET30-ORF6-Rv	GCTTCCTTTCGGGCTTTG	Sequencing pET6Ap derivatives
p55-Fw	GCGTTCTTCGGTTGGGAAC	Sequencing pCVF2
MmpL7-Fw1	GATCGTGCTGCTGACCGC	Sequencing pCVF7
MmpL7-Fw2	ATCGACACAGACAATGAGCG	Sequencing pCVF7
MmpL7-Rv2	CGTCCTGGATGGTTGTTTC	Sequencing pCVF7
P55- trans-in	ACGCCCTGGCCGAACAGC	Sequencing pCVF2
MmpL7-30aORF6-F	ATGCCTAGTCCGGCTGGC	To clone *mmpL7* gene in pET6Ap.
Tap-pT7-7-F	TTTTCATATGCGCAACAGCAACCGCGGCCCGGCATTC	To clone *tap* gene in pT7-7. *Nde*I site underlined.
P55-pT7-7-F	TTTTCATATGCGCGCGGGCCGCCGCGTGGCGATTAGCGCGGGCAGCCTG	To clone *P55* gene in pT7-7. *Nde*I site underlined.
Tap-pT7-7-R	TTTTAAGCTTATTCACTGAGCCGATCCT	To clone *tap* gene in pT7-7. *EcoR*I site underlined
P55-pT7-7-R	TTTTAAGCTTGGTGGCTCGTTAGAGCGGCTCC	To clone *P55* gene in pT7-7. *EcoR*I site underlined
pT7-7-Fw	CATGTTTGACAGCTTATCATCGATG	Sequencing pT7-7 derivatives
pT7-7-Rv	GGGAGACCACAACGGTTTC	Sequencing pT7-7 derivatives
MmpL7-30aORF6-R	GTCTCGAACAGGCGGTCAAC	To clone *mmpL7* gene in pET6Ap.

Table 17. Oligonucleotides used in construction and verification of plasmids.

Oligonucleotides used for allelic exchange purposes		
Oligonucleotide	**Sequence 5'->3'**	**Description**
uppOlmmpL7	TGCTCAGCCTGAACAGTCCAAGTCTTAACTTAAACAGGAGCCGTTAAGACatgcctagtccggctggccgtcta	To generate CVF13 strain. Used to amplify *mmpL7* gene with homology arms (shown in capital letters), which are homologous to the 50 pb regions flanking *acrB* gene.
lowOlmmpL7	GTTATGCATAAAAAAGGCCGCTTACGCGGCCTTAGTGATTACACGTTGTAtcaacgccgcccctggcgtggtcgg	
repOlacrB615-28mmpl	GAACAACGTTGAGTCGGTGTTCGCCGTTAACGGCgccggggcaaggcaagcacagcaatacctcgatcccatgcTCGTTTCCTTGAAGGACTGGGCCGATCGTCCGGGCG	To generate CVF19 strain. Capital letters indicate the homology arms that hybridize within *acrB* gene. The region belonging to *mmpL7* gene is shown underlined.
acrB S 5-f	CCTTCTTGCCAGATGAGGAC	To confirm the replacement of the *rpsL-neo* cassette by repOlacrB615-28mmpl oligonucleotide. Used to verify CVF19 strain by sequencing.
acrB S 5-r	GCAGTACCCAGTTCCACGAT	

Table 18. Oligonucleotides used in allelic exchange techniques.

Plasmid DNA extraction (minipreparation)

Plasmid DNA extractions were done with QIAprep Spin Miniprep Kit (Qiagen), following manufacter's instructions. *E. coli* strains were cultured overnight either in LB liquid medium on LB agar plates; for extraction 1.5 ml liquid culture or culture from plate were used.

Plasmidic DNA was kept at -20ºC.

Construction of plasmids

We used two expression vectors for *E. coli*: pT7-7 (70) and pET30a-ORF6 (43).

Construction of vectors for expressing Rv1258c *and* Rv1410c *efflux pumps from M. tuberculosis.*

The PCR amplifications for cloning were performed with a high fidelity DNA Polymerase (TripleMaster® PCR System (Eppendorf)). The oligonucleotides used in this PCRs were designed with the proper restriction sites.

Since the codon usage in *E. coli* is different from the one of *M. tuberculosis* (7), we changed the first codons of *Rv1258c* and *Rv1410c* genes in order to optimise the expression of these mycobacterial efflux pumps in *E. coli*. With that aim, we designed primers with nucleotide substitutions so that they were adapted to the *E. coli* codon usage, and led to no change in the codified aminoacid (Fig 26).

A. *Rv1258c*

```
       5'-ATG AGA AAC AGC AAC CGC GGC CCG GCA TTC ctg atc ctg ttc gca...-3'  M. tuberculosis Rv1258c gene
          Met Arg Asn Ser Asn Arg Gly Pro Ala Phe Leu Ile Leu Phe Ala        aa sequence

5'-ttttcatATG CGC AAC AGC AAC CGC GGC CCG GCG TTT ctg atc ctg ttc gca -3'   primer "Tap-pT7-7-F"
          Met Arg Asn Ser Asn Arg Gly Pro Ala Phe Leu Ile Leu Phe Ala        aa sequence
```

B. *Rv1410c*

```
       5'-ATG CGA GCA GGA CGT CGA GTC GCG ATT AGC gcg ggc agc ctg...-3'  M. tuberculosis Rv1410c gene
          Met Arg Ala Gly Arg Arg Val Ala Ile Ser Ala Gly Ser Leu        aa sequence

5'-ttttcatATG CGC GCG GGC CGC CGC GTG GCG ATT AGC gcg ggc agc ctg -3'   primer "P55-pT7-7-F"
          Met Arg Ala Gly Arg Arg Val Ala Ile Ser Ala Gly Ser Leu        aa sequence
```

Fig. 26. Forward primers for amplification of *Rv1258c* and *Rv1410c* genes adapted to codon usage of *E. coli*. Nucleotide substitutions are highlighted in yellow. The recognition site for *Nde*I restriction enzyme is written in blue and underlined.

A 1279 pb fragment containing *Rv1258c (tap)* gene of H37Rv was amplified from pCRS4 plasmid as a template (see Chapter 1), using oligonucleotides Tap-pT7-7-F and Tap-pT7-7-R. PCR product was digested with enzymes *Nde*I and *Hind*III and ligated with the vector pT7-7 previously linearized with *Nde*I and *Hind*III, yielding the plasmid pCVF1. The plasmid was sequenced with the oligonucleotides pT7-7-Fw, pT7-7-Rv and ctap3 (Table 17) to confirm the insert.

In order to generate pCVF2, a 1583 bp fragment containing *Rv1410c* (*P55*) gene from *M. tuberculosis* H37Rv was amplified by PCR from pCVZ1 (see chapter 1) with oligonucleotides P55-pT7-7-F and P55-pT7-7-R. This PCR fragment was digested with the restriction enzymes *Nde*I and *Hind*III and inserted into pT7-7 vector digested with *Nde*I and *Hind*III. The resulting plasmid was sequenced with oligonucleotides pT7-7-Fw, pT7-7-Rv, P55-Fw and P55-trans-in to verify the insert (Table 17).

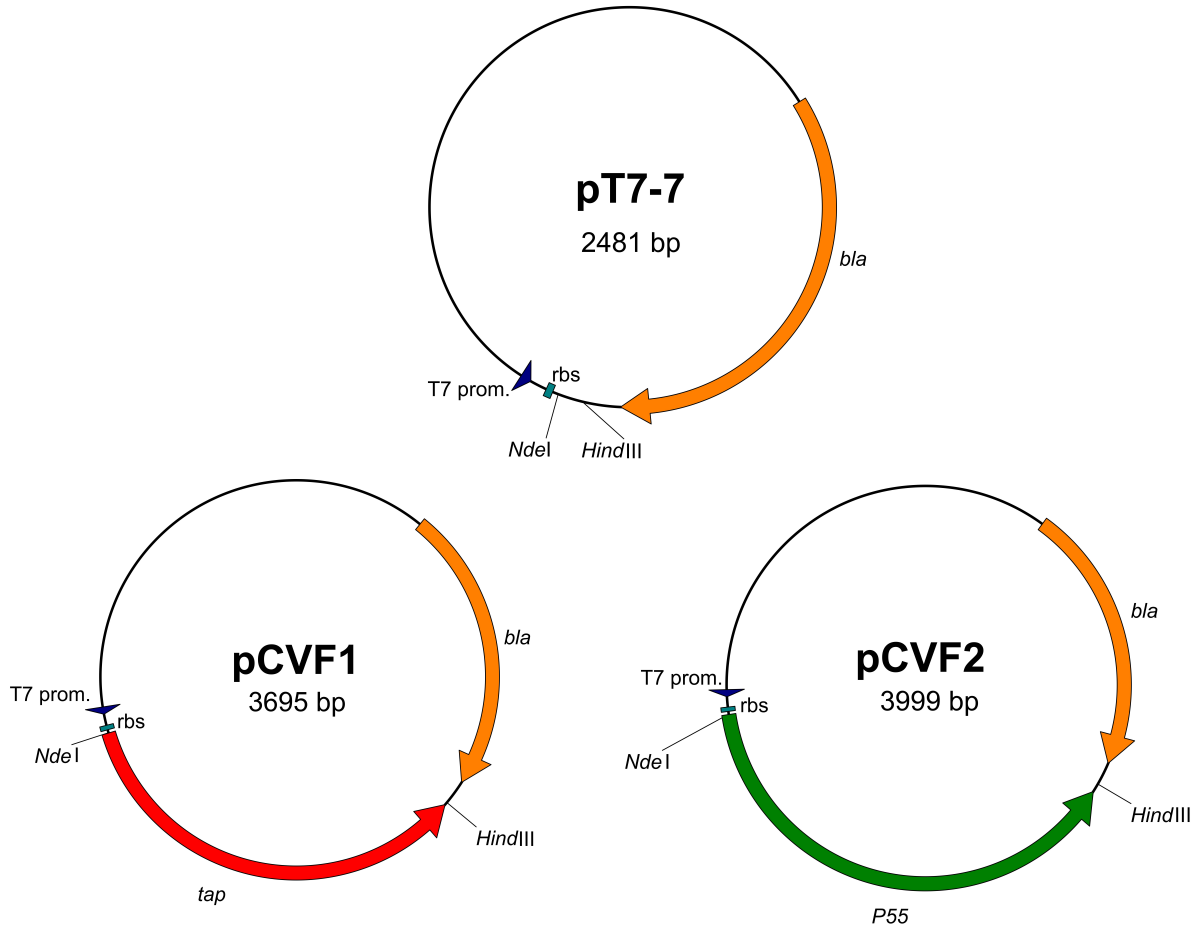


Fig. 27. pT7-7 vector and derived plasmids pCVF1 and pCVF2

Construction of vectors for expressing MmpL7 efflux pump from M. tuberculosis

We generated a bicistronic plasmid that didn't carry a km^R gene, so it could be used for expression of mycobacterial genes in Km-resistant *E. coli* strains. First, the *Ω-Ap* cassette, was released from plasmid pKT254Ω-Ap (34) by digestion with *Sma*I restriction enzyme, and subsequently inserted in the *Sma*I site of the Km^R gene in pET30a-ORF6 (43). The resulting plasmid, pET6Ap, was checked by restriction analysis with *Sma*I. The orientation of the *Ω-Ap* cassette was not investigated.

In order to construct pCVF7, a 7841 bp fragment containing *mmpL7* gene from H37Rv was amplified using oligonucleotides MmpL7-30aORF6-F and MmpL7-30aORF6-R, with Phusion High-Fidelity DNA Polymerase (New England Biolabs), which leaves the product with blunt ends. As a template, BAC-Rv30 (which contains from 3281413-3356541 nucleotides from H37Rv genome) was used. The plasmid pET6Ap was linearized with *EcoR*V, yielding a blunt-ended fragment, and subsequently ligated with the insert. To select the correct orientation of the insert in the resulting plasmid, two PCRs were performed: i) pET30-

ORF6-Fw and MmpL7-Rv , ii) pET30-ORF6-Rv vs MmpL7-Fw2 (Table 17). The plasmid was then sequenced with pET30-ORF6-Fw, pET30-ORF6-Rv, MmpL7-Fw1, MmpL7-Fw2, MmpL7-Rv and MmpL7-Rv2 (Table 17), to confirm the absence of mutations in the insert.

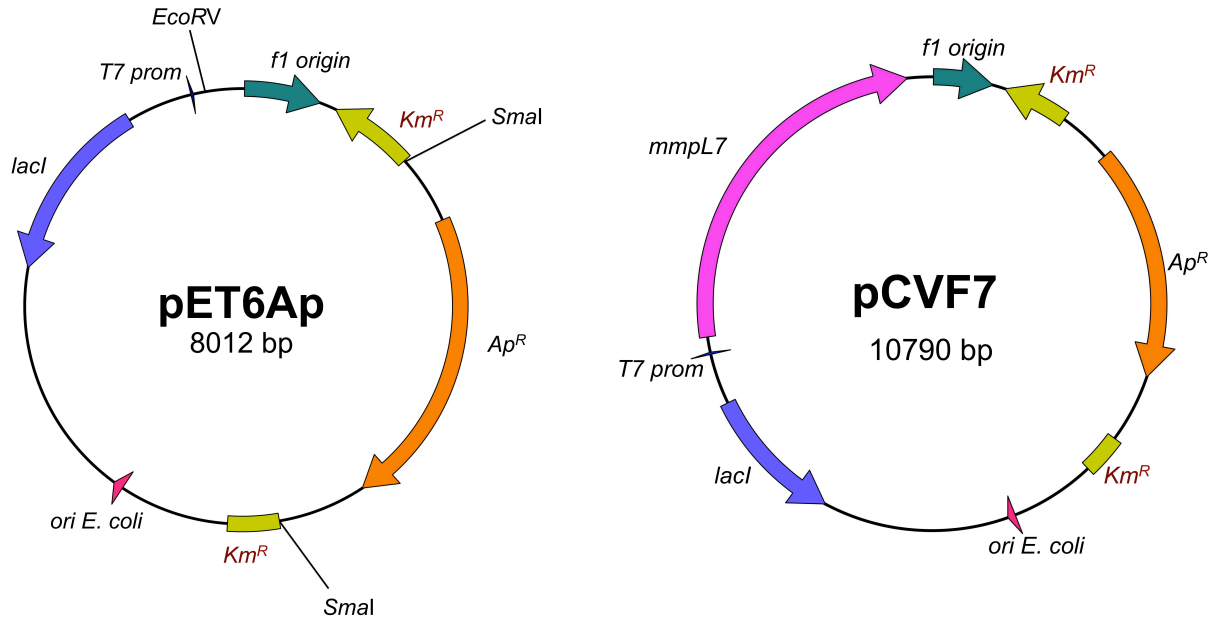


Fig 28. A. pET6Ap, derived from pET30a-ORF6, the Km^R gene is inactivated by the *Ω-Ap*^{R} cassette (orientation is not known). B. pCVF7 plasmid, for expression of *mmpL7*.

Plasmids			
Plasmid	**Marker genes**	**Description**	**Ref.**
pT7-7	Ap	Vector for expressing genes under control of T7-promoter in *E. coli*.	(70)
pCVF1	Ap	pT7-7 derived plasmid with *Rv1258c (tap)* gene, under control of T7 promoter.	This work
pCVF2	Ap	pT7-7 derived plasmid with *Rv1410c (P55)* gene under control of T7 promoter.	This work
pET30a-ORF6	Km *(Tn903*- derived)	Vector for expressing genes under control of T7-promoter in *E. coli*, upon induction with IPTG.	(43)
pET6Ap	Ap	Derived from pET30a-ORF6, with *Km*^{R} inactivated by insertion of *Ω-Ap*^{R} cassette.	This work
pCVF7	Ap	pET6Ap derived plasmid with *mmpL7* gene under control of T7 promoter, upon induction with IPTG.	This work

Table 19. Plasmids used in this study

Generation of *E. coli* competent cells & electroporation

To prepare electrocompetent cells, 150 ml of a bacterial culture were grown to an OD_{600nm} of 0.4. Then the growth was stopped for 30 min on ice, and bacteria were washed twice in chilled-cold water, and once in chilled-cold 10% glycerol. Cells were finally resuspended in 1 ml chilled-cold 10% glycerol. Aliquots of 40 µl can be storaged at -80ºC for further use.

Alternatively, if cells are to be electroporated inmediately, a simplified protocol can be used. Independent aliquots of 1.4 ml LB, pre-warmed at 37ºC, were inoculated with 30 µl fresh *E. coli* culture, and incubated at 37ºC 1000 rpm until they reached an OD~0.4. Growth was then stopped by placing the cells on ice for 2 minutes. Subsequently, bacteria were pelleted (11000 rpm, 30s, 2ºC) and two washes with 1ml chilled-cold water were performed. Finally, the pellet was resuspended in 30-40 µl chilled-cold water and immediately electroporated.

Electroporation

Aliquots of 40 µl were electroporated with 1 µl purified plasmid (~100 ng) DNA in 0.1 cm gap cuvettes (Bio-Rad) with a single pulse (1,8 kV) in a E. coli Pulser™ (Bio-Rad). Cells were resuspended in LB to a final volume of 1 ml and incubated for 1h at 37ºC 1000 rpm before plating several dilutions on plates containing the needed antiobiotic. Colonies appeared after incubation overnight.

Construction of mutants by Allelic Exchange in *E. coli*

These method is based in the enhanced recombination frequency by external recombinase enzymes, belonging to λ-phage. We used the Counter Selection BAC Modification Kit (Gene Bridges). This kit contains the plasmid pRedET, which carries the λ-phage *red*$\gamma\beta\alpha$ operon expressed under the control of the arabinose-inducible pBAD promoter, it has a thermosensitive origin of replication and confers tetracycline resistance.

The homologous recombination event will take place between a targeted gene of the bacterial chromosome (*acrB* gene in this case) and a linear DNA fragment flanked by two homology arms. Each homology arm is 50 bp long, and they will determine the region where the homologous recombination will occur. In a first step of recombination, a *rpsL-neo* cassette, conferring resistance to Km and susceptibility to Sm, flanked by these homology arms, is inserted. The final construction is obtained by recombination of a DNA linear fragment that replace the *rpsL-neo* cassette.

This fragment was obtained by PCR, using a high fidelity DNA Polymerase (TripleMaster® PCR System (Eppendorf)) and oligonucleotides designed with the correspondent homology arms (Table 18). Subsequently, this PCR product was precipitated with 3 volumes of EtOH and 0,1 volumes of NaAc 3M, incubated at -80ºC for 5 min and then pelleted and resuspended to a final concentration 0,5-1 µg/ml.

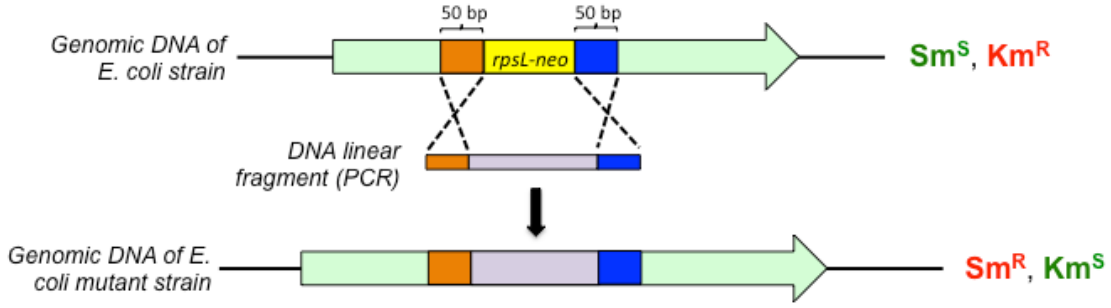


Fig.29. Schematic representation of the allelic exchange replacement for generating mutants of *E. coli.*

The *rpsL-neo* cassette had been previously introduced into the *acrB* gene of strain 3AG100 (20). First, pRedET was transformed in the selected *E. coli* strain. All the incubations must be done at 30ºC in order to keep this themosensitive plasmid. The next step was the electroporation of the linear DNA fragment. For this, 30 µl of culture grown at 30ºC overnight were used to inoculate an aliquot of 1.4 ml LB/Tet broth. When this culture reached OD_{600nm}=0.2-0.3, 50 µl L- arabinose 10% were added, and further grown at 37ºC in order to induce the expression of recombinases, until OD_{600nm}=0.4. Growth was then stopped by placing the cells on ice for 2 minutes. Subsequently, bacteria were pelleted (11000 rpm, 30s, 2ºC) and two washes with 1ml chilled-cold water were performed. Finally, the pellet was resuspended in 30-40 µl chilled-cold water and immediately electroporated with 1 µl purified plasmid (0,5-1 µg DNA) in 0.1 cm gap cuvettes (Bio-Rad) with a single pulse (1,8 kV) in a E. coli Pulser™ (Bio-Rad). Cells were resuspended in LB to a final volume of 1 ml and incubated for 1h at 37ºC 1000 rpm. The strains resulting from the homologous recombination have lost the *rpsL-neo* cassette, thus they are Km susceptible and Sm resistant; because of this the transformation mix was plated on LB Sm and incubated at 37ºC overnight. The thermosensitive plasmid pRedET was eliminated by incubating at 37ºC. Several PCRs were performed in order to verify the generated strains.

Reagents & solutions

NaAc: NaAc 3M pH 5,3

Susceptibility assays

E. coli

Serial two-fold dilutions of antibiotics were performed in LB medium in 96-well microtiter plates, with a final volumen of 50µl per well. Cultures grown overnight on agar LB plates were used to prepare a cell suspension in NaCl 0,9%, and turbidity was adjusted to 0.5 McFarland standard. This suspension was diluted 1/50 and use to inoculate the antibiotic plate, with a volumen of 50 µl per well. Results were observed after 15-18h of incubation at 37ºC. Minimal Inhibitory Concentration (MIC) was the one of the first well with no visible bacterial growth.

M. tuberculosis

Serial two-fold dilutions of antibiotics were performed in 7H9/glycerol 0.5%/OADC medium, in 96-well microtiter plates, with a final volumen of 100µl per well. Liquid cultures in logarithmic phase were adjusted to OD_{600nm} ~ 0.25 and diluted 1/100 in 7H9/glycerol 0.5%/OADC. 100µl of this suspension were added to each well and plates were incubated 6 days at 37ºC. 30 µl of a resazurin solution 0,1 mg/ml were added to each well, and results were observed after 48h of incubation at 37ºC. Resazurin (blue) is an indicator of bacterial growth, because metabolic activity of bacteria reduces it to resofurin (pink). Minimun Inhibitory Concentration (MIC) is the concentration of antibiotic of the first well that doesn't change color from blue to pink.

Solutions & Reagents

Resazurin: 0,1 mg/ml resazurin in distilled water

Accumulation assays

We performed acummulation assays with ethidium bromide (EtBr) (18, 71), phenyl-arginine-ß-naphthylamide (PAßN) (19) and pyronin Y (Pyr) (89), in presence and absence of efflux inhibitors, such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), PAßN and 1-(1-naphthylmethyl)-piperazine (NMP).

The intracellular accumulation of these compounds was estimated by measuring fluorescence intensity. Each compound tested has different characteristics that make them suitable for this assay: EtBr increases its fluorescence when its intercalated in the DNA; PAßN is cleaved by intracellular esterases to yield the highly fluorescent metabolite ß-naphthylamine; and fluorescence of Pyr is quenched after binding to RNA.

An overnight culture on agar LB plate was used to make a suspension in buffer (PBS 1x + 0,4% glucose). O.D. $_{600nm}$ was adjusted to 1 and 200 µl of this suspension transferred to each well of a opaque black 96-well plate.

The substrate of accumulation was then added to the cell suspension to the corresponding final concentration (EtBr 25µM, PAßN 200µM, Pyr 2,5µM) and, if necessary, the efflux inhibitor to a final concentration of 200µM. Wild-type and Δ*acrB* strains were included as controls.

Relative fluorescence intensity was measured over time in a Safire (Tecan, Crailsheim, Germany) fluorescence plate-reader. Selected wavelengths for measuring each compound are listed in Table 20.

Compound	λ. Excitation Max. (nm)	λ Emission Max (nm)
EtBr	518	605
PAßN	320	460
Pyr	545	570

Table 20. Wavelength settings for compounds used in accumulation

Real-time accumulation and efflux assays

First, we prepared a bacterial suspension to perform these assays. For this, a 20 ml LB medium was inoculated directly from cryovials of from an overnight culture on agar LB plates. This culture was incubated overnight (18-20h) at 37ºC and shaking (200 rpm), thus it will reach stationary phase and starvation of nutrients. 10 ml of this stationary culture were transferred to a 50ml tube and washed with 20 ml PPB buffer (20mM K_3PO_4, 1 mM $MgCl_2$, pH7). Then, it was centrifuged (5 min at 4000rpm), supernatant removed and pellet resuspended in 30ml of PPB buffer. With the same buffer it was adjusted to an OD_{600}= 0.25. This cell suspension was used to perform two different experiments:

a) Real time efflux assay
b) Real time accumulation & efflux assay

These assays must be performed with AcrAB-TolC substrates fulfilling a very important characteristic: they must be strongly fluorescent in a non-polar environment, and its fluorescence decreased drastically in a polar environment. Because of this, we are able to distinguish the compound inside the bacteria from the one outside. In our study, we used

1,2-Dinaphthylamine (1,2-DNA) (21) and BM-27; both are highly lipophilic and consequently they adhere to plastic, because of this all the tubes must be made of glass.

Real time efflux assay

2 ml of bacterial suspension previously prepared were transferred to a glass tube, CCCP added to a final concentration of 5-20 μM (depending on the *E. coli* strain) and incubated for 15 minutes to de-energize the cells. 1,2'-DNA was added to a final concentration of 16 μM; it enters the cell by passive difussion, but the bacterium is not able to export it by active efflux, thus resulting in the accumulation inside the cell. The glass tube was sealed with film and incubated 2-3 h at room temperature. As a result, bacteria were loaded with 1,2'-DNA.

When the incubation time was over, CCCP and 1,2'-DNA that didn't enter into bacteria were removed by washing the pelleted bacteria with 2 ml of PPB. This suspension was transferred to a quarzt cuvette and placed into a spectrofluorometer (Perkin Elmer LS55). By measuring the fluorescence we were informed of the efflux of the substrate in real time.

At second 100 of experiment, pre-loaded bacteria were stimulated with glucose at a final concentration of 50 mM. Glucose energizes the cells, which are now able to actively export the substrate through their transporters. This phenomenon was translated into a decrease of fluorescence.

Real-time accumulation and efflux assay

2 ml of the cell suspension were transferred to a quartz cuvette and inmediately placed in the spectrofluorimeter to start measurement. After 50 s, CCCP was added to a final concentration of 10 μM; due to quenching effect, fluorescence signal was reduced. At 500 s from the start of the experiment, BM-27 was added to a final concentration of 10 μM and as a consequence of accumulation inside the bacteria, fluorescence started to raise. At second 1000, glucose was added to a final concentration of 50 mM in order to energize the cells; in response a fluorescence decrease was observed, as a consequence of the active efflux of BM-27.

Dye	λ. Excitation Max. (nm)	λ Emission Max	λ 2nd Excitation Max (nm)
1,2'-DNA	370	460	810
BM-27	400	457	873

Table 21. Wavelength settings for dyes used in real time efflux and accumulation

Phenotypical characterisation of *M. tuberculosis*

Growth in liquid medium

In order to characterize growth in liquid medium, 1ml of a culture in logarithmic phase adjusted to OD_{600}=0.25 was inoculated in 50 ml of 7H9/Tween/OADC. OD_{600} was measured for 1 month. When OD_{600} reached 1, the culture was diluted ½ and ¼ and OD corrected atending the dilution. In addition, on days 4, 8 and 16 of growth, serial dilutions were plated to find the number of viable bacteria corresponding to each OD measurement.
Morphology of colonies was evaluated at 2-4 weeks of growth on solid medium.

Ziehl-Neelsen Stain

250 µl of liquid culture in logarithmic phase were pelleted, and mycobacteria inactivated with 350 µl paraformaldehyde 4% for 30-35 min at room temperature. They were washed with 150 µl PBS + tween 0,05% and staining was performed as described in literature (6).

Escherichia coli strains			
Derived from **3AG100**	**Resistance marker**	**Description**	**Ref.**
3AG100	-	AG100 derived strain overexpressing *acrB* gene	(47)
3AG100 Δ*acrB* (St. 320)	km	3AG100 *acrB*::*rpsLneo* 615-628	(20)
3AG100 Δ*acrB* (St. 513)	km	3AG100 *acrB*::*rpsLneo*	(20)
3AG100 Δ*acrB* (St. 540)	none	3AG100 Δ*acrB*	(20)
CVF13	Ap	3AG100 *acrB::mmpL7*	This work
CFV19	Ap	3AG100 *acrB*615-28::*mmpL7* 535-48	This work
Derived from **BW25113**	Resistance marker	Description	**Ref**.
BW25113 (wild type)	-	Wild type strain	(97)
Δ*acrB*	-	BW25113 Δ*acrB*	(97)
Δ*emrE*	-	BW25113 Δ*emrE*	(97)
Δ*mdfA*	km	BW25113 Δ*mdfA:: km*	(97)
Δ*acrB*Δ*emrE*	-	BW25113 Δ*acrB* Δ*emrE*	(97)
Δ*emr*EΔ*mdfA*	km	BW25113 Δ*emrE* Δ*mdfA::km*	(97)
Δ*acrB*Δ*emrE*Δ*mdfA*	km	BW25113 Δ*acrB* Δ*emrE* Δ*mdA::km*	(97)
Δ*smr*	-	BW25113 Δ*emrE* Δ*mdtIJ* Δ*sugE*	(97)
CVF1	Ap	BW25113 pT7-7	This work
CVF2	Ap	BW25113 pCVF1	This work
CVF3	Ap, Km	ΔemrEΔmdfA pCVF1	This work
CVF4	Ap, Km	ΔacrBΔemrEΔmdfA pCVF1	This work
CVF5	Ap, Km	ΔmdfA pCVF1	This work
CVF6	Ap, Km	ΔmdfA pCVF2	This work
CVF7	Ap	ΔemrE pCVF1	This work
CVF8	Ap	ΔemrE pCVF2	This work
CVF9	Ap, Km	ΔemrEΔmdfA pCVF2	This work
CVF10	Ap	ΔacrB pCVF1	This work
CVF11	Ap	ΔacrB pCVF2	This work
CVF12	Ap	ΔacrBΔemrEΔmdfA pCVF2	This work
CVF14	Ap	ΔacrB pT6Ap	This work
CVF15	Ap	ΔacrB pCVF7	This work

Table 22. *E. coli* strains used in this study

Mycobacterium tuberculosis strains		
Derived from **CDC1551**	Resistance marker	Description
CDC1551	-	Wild type strain
MmpL7KO	Km	CDC1551 Δ*mmpL7::km*
MmpL10KO	Km	CDC1551 Δ*mmpL10::km*

Table 23. *M. tuberculosis* strains used in this study

Results

Expression of efflux pumps from *M. tuberculosis* in *E. coli*

Two strains of *E. coli* were used: BW25113 and 3AG100, both derived from *E. coli* K-12. 3AG100 is a strain generated by repeated exposure of AG100 to a fluoroquinolone (47), and it overexpresses AcrAB-TolC efflux pump. Knock-out mutants Δ*mdfA*, Δ*emrE* and Δ*mdfA*Δ*emrE* were available for *E. coli* BW25113 (97). The knock-out mutant Δ*acrB* was available for BW25113 and 3AG100 (20, 97).

There are morphological differences between BW25113 and 3AG100; the first one forms bigger colonies. It has been described that BW25113 is more resistant to ethidium bromide than most of the *E. coli* strains (97). Consistenly, in our study we have found that bigger concentrations of efflux inhibitors are requiered to de-energize bacteria in the case of BW25113.

Generation of *E. coli* strains containing *M. tuberculosis* efflux pumps genes in expression vectors for *E. coli*.

In order to generate *E. coli* strains expressing efflux pumps from mycobacteria, plasmids pCVF1 and pCVF2 were transformed by electroporation in *E. coli* strains: BW25113 Δ*mdfA*, BW25113 Δ*emrE* and BW25113 Δ*mdfA* Δ*emrE*. pCVF7 was transformed in *E. coli* BW25113 Δ*acrB* and 3AG100 Δ*acrB*. (Table 22)

Generation of *E. coli* strains containing complete or partial *M. tuberculosis* efflux pumps genes in its chromosome.

Allelic replacement was used to generate *E. coli* strains containing either the whole or part of the gene *mmpL7* in its chromosome. The first step of the process, the insertion of *rpsL-neo* in the targeted region, had been made previously (20).

In order to obtain CVF13, a 3AG100-derived strain with the gene *mmpL7* from *M. tuberculosis* H37Rv replacing *acrB*, a 2863 bp fragment was amplified from *M. tuberculosis* H37Rv DNA with oligonucleotides uppOlmmpL7 and lowOlmmpL7. This DNA linear fragment was electroporated in 3AG100 Δ*acrB* (St.513) (table 22) containing pRedET plasmid, after induction of the recombinases. Colonies with phenotype Sm resistant and Km sensitive

were selected. The presence of *mmpL7* gene was checked by PCR with oligonucleotides mmpL7-Fw1 and mmpL7-Rv2, which yields a 1235 pb fragment.

The same method was used to create a hybrid *acrB-mmpL7* gene. The susbtrate binding site of AcrB has been described previously (20), and our aim was to replace a key region of its binding site by the hypothetically analogous region of MmpL7. This region comprised aminoacids 615-628 of AcrB, and was substituted by aminoacids 535-548 of MmpL7 protein. The objective of this hybrid mutant was to discover if, when part of the substrate binding site was replaced by the one of MmpL7, the pump was still functional and, if so, the substrate profile transported was different. In this case, since the region to be replaced was small, the linear DNA fragment was not a PCR product, but a 110 bp oligonucleotide, repOlacrB615-28mmpl (Table 18). This was transformed in the 3AG100 Δ*acrB* (St. 320) strain containing pRedET plasmid, as described. Colonies with phenotype Sm resistant and Km sensitive were selected. The replacement of the *rpsL-neo* cassette was verified by PCR and further sequenced with the same pair of oligonucleotides (Table 24). The resulting strain was called CVF19.

	PCR product size	
Oligonucleotides	3AG100 Δ*acrb* (St. 320)	CVF19
acrB S 5-f *vs*. acrB S 5-r	1625 bp	346 bp

Table 24. Verification by PCR of CVF19 strain, containing a hybrid *acrB::mmpL7* gene

Susceptibility assays of *E. coli* strains with *M. tuberculosis* efflux pumps genes

One of our main objectives was to find if certain efflux pumps from *M. tuberculosis* can be expressed and functional in *E. coli*. With that aim, we compared levels of antibiotic resistance of *E. coli* strains with efflux pumps genes deleted, with and without a vector containing an efflux pump gene from *M. tuberculosis*.

Rifampicin	Rifaximin
Streptomycin	Spectinomycin
Gentamicin	Tetracycline
Minoclycine	Tigecycline
Levofloxacin	Moxifloxacin
Ciprofloxacin	Clarithromycin
Erythromycin	Clindamycin
Chloramphenicol	Linezolid
Novobiocin	

Table 25. Antibiotics tested in susceptibility assays of *E. coli* strains.

No significant differences were observed in the MICs of the "complemented" mutants, nor in the case of MFS proteins neither in RND transporters. One of the possible explanations for this fact is that efflux pumps are not being correctly expressed from expression vectors. That is the reason why we constructed another expression strain for MmpL7, this time replacing the *acrB* gene by *mmpL7* gene in the chromosome of *E. coli* 3AG100. Resistance profile of this new strain was coincident with the one of the knockout mutant Δ*acrB*.

Finally, a hybrid gene *acr*B-*mmp*L*7* was generated. This gene consisted of the entire *acrB* gene, replaced only in a small but very important region belonging to the substrate binding site. This region was changed by the hypothetically homologous region of MmpL7 transporter, that was located by alignment of the aminoacidic sequences. The hybrid mutant showed the same MICs than the knock-out mutant Δ*acrB*, thus we inferred that the hybrid proteins was not functional, or it wasn't being expressed by the bacteria.

Accumulation assays

As a result of the "complementation" of the knock-out mutants of *E. coli* with efflux pumps from *M. tuberculosis*, we would expect that, if those transporters were functional, the accumulation was lower for them than for the knock-out mutant.

In none of the cases, neither for MFS nor RND proteins, we found the expected result, because the generated strains behaved the same way as the knock-out mutant. It must be noted that, for the *E. coli* strains expressing Rv1258c from *M. tuberculosis*, we found a difference in the accumulation of PAßN in presence of the efflux inhibitor NMP, but this was a raise in the accumulation, not a reduction.

Accumulation assays for EtBr and PAßN revealed that 3AG100 strain has a higher level of accumulation that BW25113, both for PAßN and EtBr.

Real time efflux and accumulation

In the host laboratory, real time efflux and accumulation assays were optimized for *E. coli* 3AG100, so the first step was to set the best conditions for the strain BW25113. Regarding real time accumulation and efflux, an increase in CCCP concentrations (from 5 µM to 20 µM) was necessary to improve accumulation; however, this accumulation didn't reach the levels of the one in 3AG100. In order to allow a better accumulation, longer times of exposition to CCCP were tried, unsuccessfully. In addition, BW25113 Δ*acrB* and Δ*acrB*Δ*mdfA*Δ*emrE* showed lower efflux levels than the wild type (Fig. 30 and 31), but still significant when compared to 3AG100 Δ*acrB* knock-out mutant.

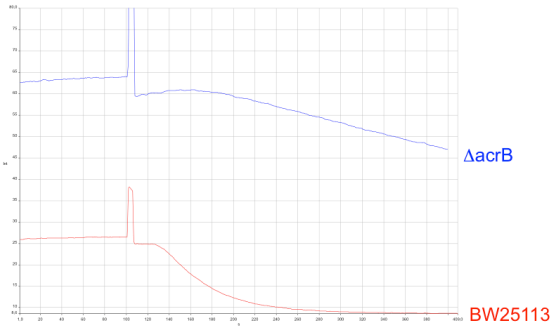


Fig.30. Real time efflux of 1,2-DNA of BW25113 and BW25113 Δ*acrB*.

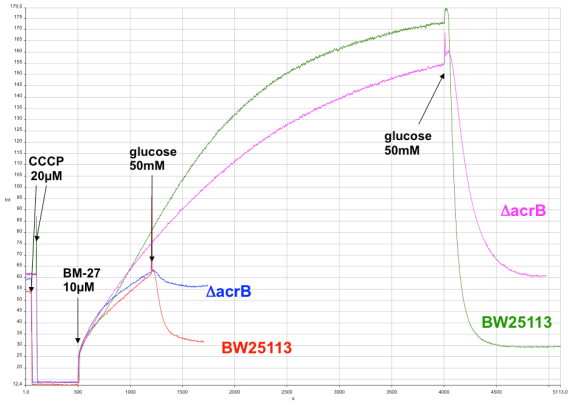


Fig.31. Real time accumulation and efflux of BM-27 of BW25113 and BW25113 Δ*acrB*.

Given the small or even no differences in efflux between BW25113 and its knock-out mutants, we concluded that it wouldn't have been possible to detect changes in the phenotype of the "complemented" knock-out mutants of *E. coli* BW25113.

The strain CVF13 (3AG100 *acrB::mmpL7*) was compared to the wild-type strain 3AG100 and 3AG100 Δ*acrB*. In both experiments, CVF13 has the same behaviour that Δ*acrB* (Fig 32 and Fig 33), so that we deduced that MmpL7 efflux pump was not being correctly expressed, or 1,2'-DNA and BM-27 are not substrates of the efflux pump.

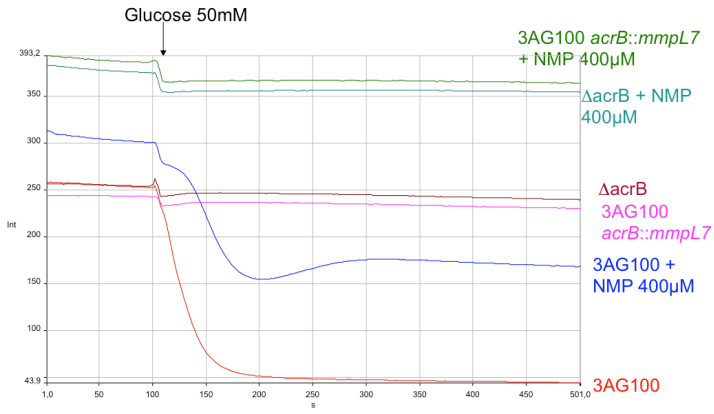


Fig. 32. Real time efflux of 1,2'-DNA of 3AG100 wild type and 3AG100 *acrB*::*mmpL7* in the presence and absence of the inhibitor NMP.

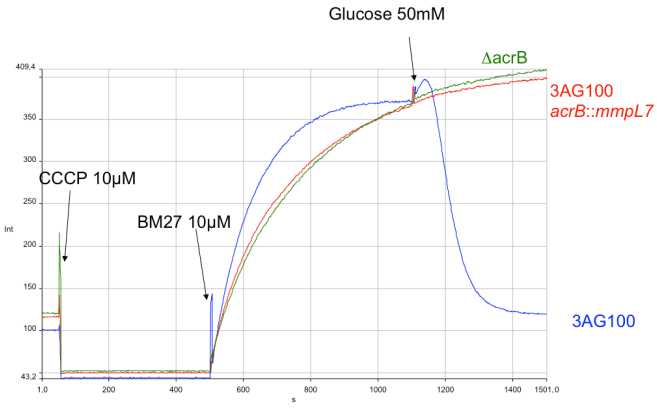


Fig. 33. Real time accumulation and efflux of 1,2'-DNA of 3AG100 wild type and 3AG100 *acrB*::*mmpL7*..

Characterization of mutants MmpL7KO and MmpL10KO of *M. tuberculosis*

MmpL7KO has less growth rate and forms less aggregates in liquid medium

MmpL7KO mutant showed phenotypical differences when compared to wild-type strain CDC1551. Firstly, MmpL7KO has an increased rate of growth in liquid medium, regarding both to OD measurement and counts of viable bacteria. At day 8 of growth, OD of the cultures of wild-type and MmpL7KO started to diverge, and from day 15 differences became more obvious. On the other hand, MmpLKO grew at similar rate as the wild-type, but slightly faster. Differences between them are clearer in the count of viable bacteria (Fig 34)

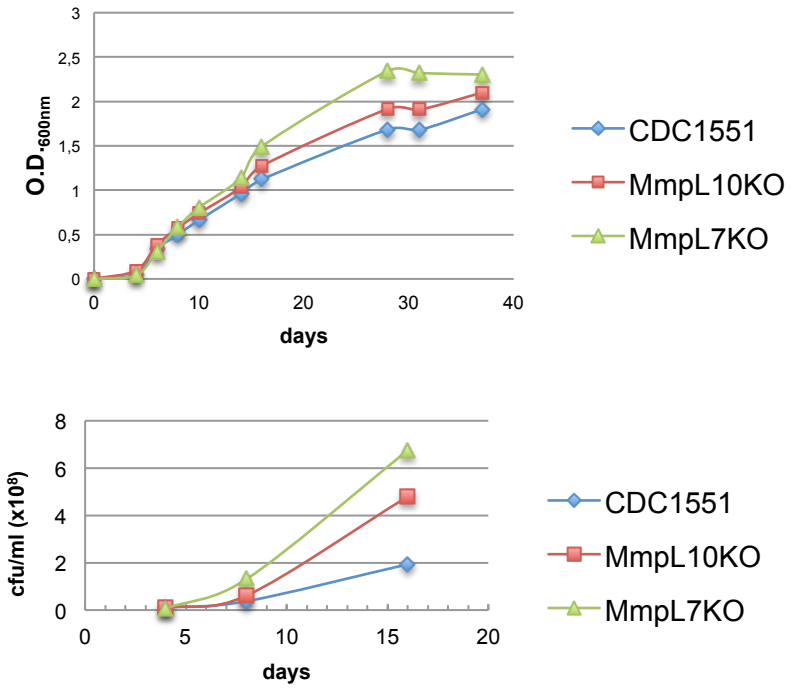


Fig.34. Characterisation of growth rates in liquid medium of CDC1551, MmpL7KO and MmpL10KO, by OD measurements and cfu count.

In addition, liquid culture of MmpL7KO forms less aggregates than the one of the wild-type strain, like it is showed in Fig. 35.

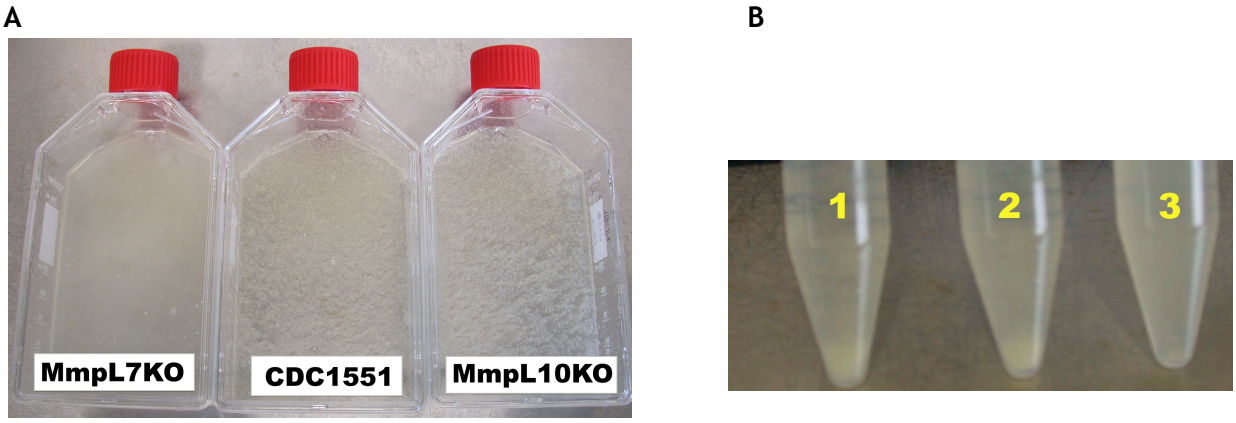
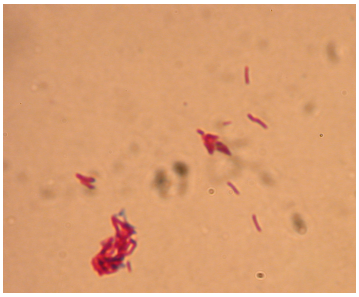


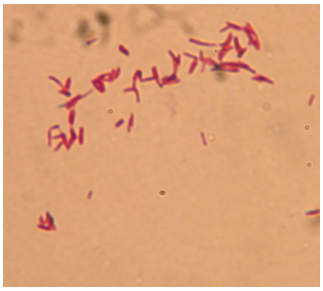
Fig 35. A. Liquid cultures of CDC1551, MmpL7KO and MmpL10KO. MmpL7KO forms less aggregates than the wild type strain. **B.** Sediment of liquid cultures of CDC1551 (n.1), MmpL7KO (n.3) and MmpL10KO (n.2). after resuspending and let them stand for about five minutes.

Zielh-Neelsen Staining

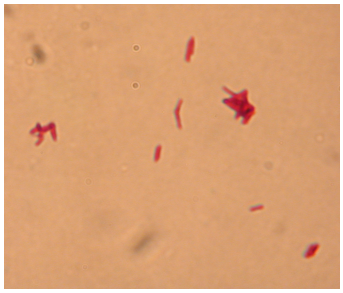
Microscope visualization of stainings did not reveal significant differences in the morphology of wild-type and mutants bacilli (Fig. 36).



CDC1551



MmpL7KO



MmpL10KO

Antibiotic susceptibility of MmpL7KO and MmpL10KO

With the aim of studying the posible contribution of MmpL7 and MmpL10 efflux pumps to intrinsic resistance of *M. tuberculosis*, we determined the MIC of MmpL7KO, MmpL10KO and CDC1551 to several compounds, including first and second line antituberculosis antibiotics.

Both mutants seemed to be more susceptible (two-fold) to tetracycline and spectinomycin, when compared to the wild-type strain. In addition, MmpL7KO showed more susceptibility to rifampicin and MmpL10KO could contribute to resistance to minoclycin. (Table 26)

Compound	CDC1551	MmpL10KO	MmpL7KO
Isoniazid	0,05-0,1	0,05-0,1	0,05-0,1
Rifampicin	0,125-0,250	0,125-0,250	0,0625
Rifaximin	<0,0625	<0,0625	<0,0625
Ethambutol	1	1	1-2
Streptomycin	0,125-0,25	0,25	0,125-0,25
Spectinomycin	64-128	32-64	32-64
Gentamicin	2	2	2
Tetracycline	8	4	4
Minocycline	2-4	1-2	2
Tigecycline	2-4	2-4	2-4
Levofloxacin	0,25	0,25	0,25
Moxifloxacin	0,125	0,125	0,0625-0,125
Ciprofloxacin	0,25-0,5	0,25	0,25-0,5
Clarithromycin	2	2	2
Erythromycin	256	256	256
Clindamycin	32-64	32-64	32-64
Chloramphenicol	2-4	4	2-4
Linezolid	0,25	0,25	0,25
Novobiocin	32	32	32
Acriflavine	2	2	2

CONCLUSIONS

- Plasmids for expressing Rv1258c and Rv1410c (MFS) efflux pumps and MmpL7 (RND) from *M. tuberculosis* have been constructed.

- *E. coli* strains containing either the whole *mmpL7* gene from *M. tuberculosis* or a hybrid gene *acrB:mmpL7* have been generated.

- No clear evidence of expression of mycobacterial efflux pumps in *E. coli* was found.

- *MmpL7* knock-out mutant of *M. tuberculosis* efflux pump has an apparently increased growth rate in liquid medium, and displayed altered colony morphology.

- Knock-out mutants of *mmpL7* and *mmpL10* seemed to be more susceptible to tetracycline and spectinomycin, when compared to the wild-type strain. MmpL7KO showed more susceptibility to rifampicin and MmpL10KO could contribute to resistance to minoclycin.

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Conclusiones generales

- La bomba de eflujo Rv1258c (Tap) de *M. tuberculosis* está implicada en resistencia intrínseca a estreptomicina, gentamicina y espectinomicina. El mutante knock-out de Tap no mostró diferencias en la susceptibilidad a tetraciclina comparada con la del wild-type H37Rv, como sí ocurría con el mutante análogo en *M. bovis* BCG. Los experimentos de infección de macrófago de ratón revelaron que Tap puede tener un papel en la virulencia de *M. tuberculosis*; sin embargo, en el modelo de infección de ratón inmunocompetente no se observaron diferencias entre el knock-out de Tap y la cepa wild type H37Rv.

- La bomba de eflujo Rv1410c (P55) de *M. tuberculosis* contribuye a la resistencia intrínseca a rifampicina, uno de los dos principales fármacos anti-tuberculosos, y a vancomicina. El mutante knock-out de P55 presenta alteraciones en el crecimiento en medio sólido: crece más despacio que la cepa wild-type, y la morfología de colonia es más plana y lisa. Los experimentos de infección de modelo celular de macrófago de ratón e *in vivo*, con ratones inmunocompetentes, mostrarons que P55 es necesaria para la virulencia completa de *M. tuberculosis*.

- El mutante knock-out de Rv2333c (Stp) no mostró diferencias en la sensibilidad a tetraciclina y espectinomicina, como sí ocurría con su análogo en *M. bovis* BCG, en comparación con la cepa wild-type. Atendiendo a los ensayos de infección en modelo de macrófago de ratón, se observó que Rv2333c podría contribuir a la virulencia de *M. tuberculosis*. sin embargo, en el modelo de infección de ratón inmunocompetente no se observaron diferencias entre el knock-out de Stp y la cepa wild type H37Rv.

- Rv1258c, Rv2333c y Rv1410c no parecen estar implicadas en la inducción de apoptosis de las células macrófago de ratón.

- Las cepas de la familia Beijing tienen una inserción (llamada Tap^{580}) de un nucleótido de citosina entre las posiciones 580 y 581 del gen Rv1258c. Esto resulta en una proteína hipotéticamente truncada. Un método PCR-RFLP que detecta esta inserción

se ha puesto a punto; permite la detección de cepas de la familia Beijing incluso desde muestras clínicas de esputo.

- Varias estrategias de expresión de bombas de eflujo micobacterianas en *E. coli* se llevaron a cabo. No se encontraron evidencias claras de expresión de estas proteínas.

- El mutante knock-out de MmpL7 tiene una velocidad de crecimiento mayor que la cepa wild-type, así como una morfología de colonia alterada.

- Aunque las diferencias en la sensibilidad fueron pequeñas, tanto el mutante knock-out de MmpL7 como el de MmpL10 resultaron ser más sensibles a tetraciclina y espectinomicina, comparando con la cepa wild-type. Además, MmpL7KO mostró mayor sensibilidad a rifampicina, mientras que MmpL10 podría estar contribuyendo a la resistencia intrínseca a minoclicina.

General Conclusions

- The efflux pump Rv1258c (Tap) of *M. tuberculosis* is implicated in intrinsic resistance to streptomycin, gentamicin and spectinomycin. The knock out mutant of *tap* didn't show a difference in susceptibility to tetraclycine compared to the wild type H37Rv strain, as it did with *M. bovis* BCG model. Infection assays in mouse macrophage cell model revealed that Tap could have a role in virulence. However, the knock-out mutant of *Rv1258c* had the same phenotype as H37Rv in infection assays in immunocompetent mouse model.

- Rv1410c (P55) efflux pump of *M. tuberculosis* is contributing to intrinsic resistance to rifampicin, one of the two main antibiotics in antituberculous treatment, and vancomycin. H37Rv knock-out mutant of Rv1410c has altered growth in solid media: it grew more slowly than the wild-type and showed a flatter and smoother morphology of colony. Mouse macrophage cell infection assays and in vivo infection (immunocompetente mice) showed that P55 has a role in virulence of *M. tuberculosis*.

- H37Rv knock-out mutant of Rv2333c *(*Stp*)* didn't show increased susceptibility to tetracycline and spectinomycin, as it did *M. bovis* BCG *Rv2333c* knock-out mutant. Infection assays in mouse macrophage cell model revealed that Stp could have a role in virulence. However, infection assays of immunocompetent mice showed no difference between the replication in lungs of the knock-out mutant of *Rv2333c* and that of H37Rv wild-type strain.

- Rv1258c, Rv2333c and Rv1410c don't seem to be implicated in apoptosis induction of mouse macrophage cells.

- Beijing strains carry a insertion of a cytosine nucleotide between positions 580 and 581 (called Tap^{580}) of Rv1258c gene. This results in a hypothetically truncated Tap protein. A PCR-RFLP method that detects Tap^{580} insertion has been developed. It allows fast detection of Beijing strains even from sputum samples.

- Several strategies to express mycobacterial efflux pumps in *E. coli* had been made. No clear evidence of expression of these efflux pump was found.

- *MmpL7* knock-out mutant of *M. tuberculosis* efflux pump has an apparently increased growth rate in liquid medium, and displayed altered colony morphology.

- Although differences in susceptibility are small, knock-out mutants of *mmpL7* and *mmpL10* seemed to be more susceptible to tetracycline and spectinomycin, when compared to the wild-type strain. MmpL7KO showed more susceptibility to rifampicin and MmpL10KO could contribute to resistance to minoclycin.