Mutagenic effect of antibiotics on *Escherichia coli* and new genes of antibiotic resistance in *Mycobacterium smegmatis*

by

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Abstract

The response of bacteria to environmental stresses including antibiotic stresses is one of the key factors helping them survive and evolve. In this thesis, we studied antibiotic resistance in two different aspects: mutagenic effect of antibiotics and mechanisms of antibiotic resistance. In many bacteria, including *Escherichia coli*, the stimulation of mutagenesis is mediated by the SOS response. Thus, blockage or attenuation of this response through the inhibition of RecA has been proposed as a possible therapeutic adjuvant in combined therapy to reduce the generation of antibiotic resistance. We studied the capacity of sublethal concentrations of antimicrobials of different families with different molecular targets, which cause the increase in the mutant frequency of *E. coli*, and the effects that the inactivation of *recA* would have on the antibiotic-mediated mutagenesis. We tested the mutagenicity of 13 antibiotics; among them eight antibiotics stimulate *E. coli* mutagenesis (slightly in most cases), with trimethoprim alone or in combination with sulfamethoxazole producing the highest impacts. Inactivation of *recA* abolishes the mutagenic effect and also produces increased susceptibility to some anti-microbials. The fact that inactivation of *recA* reduces mutagenicity and/or increases the activity of a large number of antimicrobials, supports the hypothesis that RecA inhibition might have favourable effects on antibiotic therapy. We also studied the responses of bacteria under the antibiotic pressure including the development of antibiotic resistance. Thus, a library of 11.000 *Mycobacterium smegmatis* insertion mutants were constructed and analyzed to find candidates which may have a rifampicin resistant phenotype. Rifampin is an important first-line antibiotic for the treatment of tuberculosis. Although most rifampin-resistant strains arise through mutations in the *rpoB* gene in mycobacteria, a proportion of such strains showed no *rpoB* mutations. This suggests that alternative mechanisms are responsible for rifampin resistance. We found that the disruption of *trkA*, a putative regulator of K^+ uptake, and the disruption of *ich*, a putative K^+ channel lead to increased rifampicin resistance. Our data indicate that TrkA and Ich are important for maintenance of the *M. smegmatis* growth rate, its pH homeostasis and membrane potential. Besides increasing rifampicin resistance, inactivation of these genes confers resistance to other hydrophobic agents, such as novobiocin, as well as increased susceptibility to isoniazid and positively charged aminoglycosides. We suggest that *trkA* and *ich* are general regulators of antibiotic susceptibility, and that changes in the multidrug susceptibility/resistance pattern detected in the *trkA* and *ich* mutants are associated with the membrane hyperpolarization.

Resumén

La respuesta de las bacterias frente el estrés ambiental, incluyendo el estrés generado por los antibióticos, es uno de los factores clave que las ayuda a sobrevivir y evolucionar. En esta tesis, hemos estudiado la resistencia a antibióticos desde dos puntos de vista distintos: El efecto mutagénico de los antibióticos y los mecanismos de resistencia a los mismos. En muchas bacterias el incremento de la mutagénesis está mediada por la respuesta SOS. Por lo tanto, se ha propuesto el bloqueo o atenuación de dicha respuesta mediante la inhibición de RecA como tratamiento adyuvante en terapias combinadas para reducir la aparición de resistencia a los antibióticos. Nosotros hemos estudiado la capacidad de concentraciones subletales de antimicrobianos de diferentes familias con distintas dianas moleculares que causan un incremento en la frecuencia de mutantes en *E. coli* y los efectos que una inactivación de *recA* tienen en la mutagénesis mediada por antibióticos. Exploramos la mutagenicidad de trece antibióticos, de los cuales ocho estimulan la mutagénesis en *E. coli* (ligeramente en la mayor parte de los casos), siendo el Trimetropin por sí solo o en combinación con Sulfametoxazol, el que produce mayor efecto. La inactivación de *recA* elimina el efecto mutagénico y además incrementa la sensibilidad a ciertos antimicrobianos. El hecho de que la inactivación de *recA* reduce la mutagenicidad y/o aumenta la actividad de un gran número de antimicrobianos apoya la hipótesis de que la inhibición de RecA podría tener efectos favorables en la terapia antibiótica.

También se ha estudiado la respuesta bacteriana en frente a la presión ejercida por antibióticos, incluyendo el desarrollo de resistencia. De esta manera, se construyó una librería de 11.000 mutantes por inserción de *Mycobacterium smegmatis*, los cuales se analizaron en busca del fenotipo resistente a rifampicina. La rifampicina es uno de los antibióticos de primera linea utilizado para el tratamiento de tuberculosis. Si bien la resistencia a rifampicina en mycobacteria está principalmente dada por mutaciones en *rpoB*, hubo fenotipos resistentes a rifampicina que no presentaron mutaciones en dicho gen. Esto sugiere que existen mecanismos alternativas responsables de de la resistencia a rifampicina. Durante el trabajo, hemos observado que la disrupción del gen *trkA*, un regulador putativo de la introducción de potasio a la célula, así como la disrupción de *ich*, un canal de potasio putativo, incrementan la resistencia a rifampicina. Nuestros resultados indican que tanto TrkA como Ich, son importantes para mantener la el equilibrio ácido-base, tasa de crecimiento y potencial de membrana en *M. smegmatis*. Además de aumentar la resistencia a rifampicina, la inactivación de estos genes confiere resistencia a otros agentes antimicrobianos de tipo hidrofóbico como la novobiocina, así como también provoca un aumento en la sensibilidad a isoniazida y a antibióticos aminoglicósidos cargados positivamente. A partir de los resultados obtenidos, sugerimos que *trkA* y *ich* regulan de manera general la suceptibilidad a antibióticos, y que los cambios en los perfiles de susceptibilidad y resistencia a múltiples antibióticos observados en las mutantes de *trkA* e *ich* se asocian a la hiperpolarización de la membrana.

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Contents

Chapter 1

Introduction

1.1 Antibiotic stress and the development of antibiotic resistance

Like the other living organisms, bacteria are continuously exposed to environmental stresses and are able to adapt themselves to severe fluctuations of the environment. There are many factors causing stresses in bacteria. Among them, the presence of antibiotics produces different types of vital stresses. Studying the evolution of the bacterial cell structure under such stressful conditions is an important research topic because it provides us with an insight of how bacteria become antibiotic resistant.

Antibiotics can provoke antibiotic resistance through mutator selection. Simple or successive selections with antibiotics for mutant phenotypes increase the proportion of mutator strains in a cell population [Mao et al., 1997]. In such cases, antibiotics used for selections do not only stimulate resistance to themselves, but also the increase of mutator strains may result in acquired resistance of a selected population to other antibiotics. Bacteria acquire resistance to antibiotics by one of two mechanisms: either spontaneous mutations or horizontal transfer. Resistance by spontaneous mutations depends on the mutation rate and the presence of proofreading and repair mechanisms [Miller, 1996]. Mutations may cause the resistance of bacteria to antibiotics by modifying their target, by changing their uptake or by stimulating an increase of their

efflux [Hooper, 2001, Normark and Normark, 2002]. This kind of resistance is usually found in bacteria that do not have any effective mechanisms of gene transferring such as *Mycobacterium tuberculosis*. On the other hand, resistance by horizontal transfer of genes is the main mechanism of acquired resistance. A horizontal gene transfer usually consists of three different basic processes: conjugation, transduction and transformation [Rice, 2000]. These processes do not only occur in the same species but also among different ones. Thus, horizontal transfers are a major reason of wide spreading resistance [Salyers and Amábile-Cuevas, 1997, Maiden, 1998].

Mutations and horizontal transfers can occur together. Indeed, in the resistance caused by genes such as extended-spectrum TEM-type β-lactamase variants, the horizontal transfer introduces new resistant alleles in a cell population. Then these alleles are modified to produce various variants of resistance by mutations [Blazquez et al., 1995, Blazquez et al., 2000].

1.2 Bacterial models used in this work

Escherichia coli: is Gram-negative, rod-shaped, flagellated, motile, oxidase negative and facultative anaerobe. It is a member of the Enterobacteriaceae family [Buxton and Fraser, 1977] usually having two types of metabolism: respiratory and fermentative, which commonly take place in the intestinal tract of humans and other animals. In 1922, *E. coli* K-12 strain was isolated [Bachmann, 1972]. This strain does not have virulence characteristics; it grows easily in laboratory media. Moreover, it has been proven to be an useful model for microbial physiology and genetic research. In one part of this thesis, we examined the role of antibiotics as mutagens in *E.coli* .

Mycobacterium smegmatis, used as a model organism in this study, is a Gram-positive bacterium with high G+C DNA which belongs to the genus *Mycobacterium*, the Mycobacteriaceae family, the Corynebacterineae suborder, the Actinomycetales order and the Actinobacteridae class. Species in the genus Mycobacterium consist of both environmental nonpathogenic organisms and obligate, opportunistic pathogenic. Mycobacteria have an unusual structure because their cell wall contains a complex of

long chains of mycolic acids and peptidoglican-arabinogalactan.

In particular, mycobacteria are usually classified to either slow or fast-growing groups. *M. smegmatis* being aerobic belongs to the latter one. Besides, *M. smegmatis* is considered as a saprophytic and non-pathogenic microorganism although it may cause skin and soft-tissue lesions [Brown-Elliott and Wallace, 2002]. Unlike most of pathogenic mycobacterial species, *M. smegmatis* is a simple model and is easy to work with because of its fast growing rate and requiring cheaper infrastructures for experiments. There are many similarities between *M. smegmatis* and the much more virulent obligate mycobacterial pathogens such as *Mycobacterium tuberculosis.* They both have the same unusual cell wall structure and have 90% genetic homology to each other [Tyagi and Sharma, 2002]. In addition to the aforementioned properties, the *M. smegmatis* mc^2 155 strain is hyper-transformable; it is an efficient host for the eclectroporation of DNA [Snapper et al., 1990] and is now the work-horse of mycobacterial genetics. Furthermore, it is readily cultivatable in most of synthetic or complicated laboratory media, where it can form visible colonies in quite a short time from just three to five days. For these reasons, *M. smegmatis* became more popular in scientific laboratories than *M. tuberculosis* or any other mycobacterial pathogens.

1.3 Antibiotics stimulate mutagenesis in *E. coli*

1.3.1 The SOS system

The SOS system is a cellular response to DNA damage; it is a programmed DNA repair which results in DNA mutagenesis and genetic exchange or recombination [Matic et al., 1995]. The SOS system is a regulon consisting of over 40 unlinked genes [Radman, 1975]. Two key regulatory proteins LexA and RecA are involved in the regulation and the induction of SOS response [Miura and Tomizawa, 1968, Defais et al., 1971]. LexA is a transcriptional repressor that has two typical domains: an N-terminal DNA-binding and a C-terminal dimerization and catalytic domain [Luo et al., 2001, Schnarr et al., 1988, Oertel-Buchheit et al., 1993]. The LexA pro-

tein binds to a site in the promoters of the SOS genes (SOS box) and down-regulates the expression of these SOS genes, including the *lexA* gene [Courcelle et al., 2001, Fernandez De Henestrosa et al., 2000]. RecA is an inducer protein of SOS response; it plays an important role in homologous recombination and in many DNA repair pathways such as repairing daughter strand gaps, double strand breaks and SOS mutagenesis. RecA is activated by binding itself to a single-stranded DNA (ssDNA) produced by DNA damages during the replication processes and forming a nucleoprotein filament in the presence of ATP [Chen et al., 2008, Cox, 2007]. The activated form of RecA , usually known as ReA*, interacts with LexA and activates the self-cleavage activity of LexA (also known as auto-cleavage) leading to the dissociation of LexA from the SOS boxes. It results in the induction of the SOS regulon [Little et al., 1980]. When a DNA damage is repaired, the activity of RecA* disappears, thus, functional LexA is re-accumulated and binds to the operator region of the SOS genes, and finally the expression of SOS genes is prevented [Erill et al., 2007].

When the SOS response is induced by DNA damage, multiple genes involved in repair, replication, recombination and cell division are expressed. Among them some important genes are listed below:

1. In nucleotide excision repair (NER): *uvrD* (helicase encoding UvrD), *uvrA* and *uvrC* encode subunits UvrA and UvrC of the UvrABC nuclease. The NER repairs any damage caused by ultraviolet radiation.

2. In replication: *polB*, *dinB* and *umuD*, *C* encode the polymerases II, IV and V respectively. These enzymes are able to bypass DNA lesions blocking chain elongation by the DNA polymerase III.

3. In recombination:

    - *recA*: encodes the RecA protein, which also have a role in SOS regulation system and is one of the key proteins involved the homologous recombination process.

    - *recN*: encodes the RecN protein involved in recombination via the RecFOR

pathway.

- *ruvAB*: encodes the RuvA and RuvB proteins, being responsible for the resolution of the holiday junctions created during homologous recombination.

4. In cell division:

   - *sulA* encodes the SulA protein, which inhibits cell division by interacting with the FtsZ protein being responsible for cell septation process [Trusca et al., 1998].
   - *ftsK*: encodes a DNA translocase that coordinates the processes of chromosome segregation and cell division [Liu et al., 1998].

1.3.2 The SOS mutagenesis

The first action of SOS response is rescuing cells via restarting replications. SOS mutagenesis is an error-prone DNA replication process allowing the bypass of DNA lesions induced by UV or other external agents via a special kind of DNA repair machinery [Walker, 1985]. Under usual conditions, the amount of LexA in a cell is enough to control the expression of the SOS genes and *lexA* itself. When auto-cleavage of LexA occurs via RecA* action, several genes from the SOS regulon are expressed to activate error-prone DNA replication and the damage is therefore repaired. When the reparation is finished, the concentration of LexA increases and the expression of the SOS genes is again inhibited. During the SOS response, three main repairing processes occur: excision, recombination and mutagenic repair.

The genes encoding DNA polymerases II (encoded by *polB*), IV (encoded by *dinB*) and V (a product of *umuD, C* genes) are expressed through the induction of SOS response. These genes known as error-prone DNA polymerases are responsible for continuing of DNA replication with DNA lesions that cannot be replicated by DNA polymerase III. This kind of replication is called translesion DNA synthesis. However, these polymerases have low fidelity on replication, thus, errors can be introduced during this process causing the generation of mutations [Sutton et al., 2000].

1.3.3 Antibiotics increase mutagenesis via the induction of SOS responses

It has been demonstrated that some antibiotics increase the mutagenesis via their ability to induce the SOS response in bacteria [Ysern et al., 1990]. Among them, quinolones work as good inducers of the SOS system. They are DNA-damaging antibiotics which can increase mutant frequency via the induction of RecA-mediated processes including also the induction of the error-prone DNA-polymerase expression [Kohanski et al., 2010]. Quinolones induce the SOS response by a mechanism in which the RecBCD nuclease/helicase is required [Newmark et al., 2005]. These drugs target DNA gyrase and form a drug-stabilized gyrase cleavage complex to inhibit the DNA replication. RecBCD processes the conversion of drug-stabilized cleavage complex into DNA breaks. This process produces ssDNA which is necessary to activate RecA to form RecA* that is required for the induction of the SOS response.

Other antibiotics being able to induce the SOS response are β-lactams. These antibiotics are cell wall synthesis inhibitors, their targets in bacteria are penicillin binding proteins (PBPs) which play an important role in the synthesis of peptidoglycan. The PBP3 protein encoded by *ftsI*, is a key element in the septation during cell division [Ishino and Matsuhashi, 1981] and is a target of β-lactams including ceftazidime. It has been demonstrated that inhibition of PBP3 by β-lactams stimulates the SOS response in *E. coli* [Miller et al., 2004] and the transcription of the *dinB* gene encoding the error-prone DNA polymerase IV [Perez-Capilla et al., 2005]. Moreover, it also has been shown that the cephalosporin ceftazidime elicits adaptive responses, including the increase of mutant frequency in *Pseudomonas aeruginosa* [Blazquez et al., 2006]. In addition, it is known that sub-inhibitory concentrations of the fluoroquinolone antibiotic ciprofloxacin promote genetic recombination in *E. coli* [Lopez and Blazquez, 2009, Lopez et al., 2007]. Recently, Kohanski et al. [Kohanski et al., 2010] have demonstrated that sublethal levels of some bactericidal antibiotics induce mutagenesis and this induction correlates with an increase in reactive oxygen species (ROS), which in turn produces an induction of the SOS response.

1.4 Anti-Tuberculosis drug resistance problem

The discovery of antibiotics is one of the most important breakthroughs in modern medicine. Since then, antibiotics have been widely used for effectively treating and avoiding many popular bacterial infections in both human and animal bodies. However, bacteria evolve very quickly to adapt themselves to new environments and become resistant to many popular antibiotics. The recent fast increase of antibiotic resistance causes a number of issues in health care. Nowadays, many well-known antibiotics quickly become ineffective making treatment processes more complicated and expensive. So far, some studies show that the main reason of antibiotic resistance is inappropriate use of antibiotics. It is shown that over-use and misuse of antibiotics can lead to the appearance and the wide-spread of antibiotic resistance.

Tuberculosis (TB) is a disease caused by several strains of mycobacteria, mainly by *M. tuberculosis*. From the beginning, anti-TB drugs have been very effective in protecting patients from tuberculosis. However, there might be unexpected side-effects of inappropriate using anti-TB drugs. On one hand, they kill bacteria but on the other hand they stimulate bacteria to become resistant to those drugs. Resistance to anti-TB drugs happens during the selection of drug-resistant mycobacteria with spontaneous mutations. When a treatment regimen is inadequate these resistant mutants widely spread and replace wild type strains. Drug resistance developing when there is no history of a TB treatment is called resistance among new cases or primary drug resistance. Similarly, drug resistance developing during or after the courses of a treatment is defined as resistance among previous treated cases or acquired drug resistance.

The wide spread of TB resistance to many locations supposses a huge challenge for many TB control programs. The increase of TB resistance is usually due to many factors including the neglects of governments in TB control, inadequate accesses and infrastructures, economic circumstances, poor managements of TB control programs, population growth, migrations and a rising number of TB cases in HIV infected individuals. Studies on drug resistance in many locations showed that the number

of drug resistant TB is much higher in developing countries than developed countries [Espinal, 2003].

Initially, bacteria are only resistant to a specific drug. However, as time goes by they accumulate mutations being resistant to every individual drug. Finally, they may become multi-drug resistance (MDR) [Ramaswamy et al., 2000]. In the literature [Iseman, 1999], MDR-TB is usually defined as TB organisms that are resistant to isoniazid and rifampicin whose are the two most potential first-line anti-tuberculosis drugs. Patients with MDR-TB are difficult to be treated effectively because it requires long-term treatments with more toxic, expensive and effective drugs. Besides, there is a high possibility that they can remain as source of infection for a long period.

Another type of TB resistance is XDR-TB defined as TB with resistance to at least isoniazid and rifampicin and resistance to a fluroquinolone or a second line injectable agent (i.e. amikacin, kanamycin or capreomycin)[for Disease Control and Prevention, 2010]. This kind of resistance is identified as among the group of dangerous resistance which results in very poor outcome and high mortality rate [Raviglione and Smith, 2007]. Both MDR-TB and XDR-TB strains are resistant to the most of important anti-TB drugs. These kinds of resistance make TB control programs more complicated.

1.5 The burden of disease caused by TB

Statistics data regarding the magnitude of the TB epidemic all over the world reported by the World Health Organization shows that one third of the world's population is infected by *M. tuberculosis*. In 2010, there were 8.8 million new infection cases; 1.1 million of deaths caused by TB infections from HIV-negative people and an additional number of 0.35 million of deaths from HIV-associated TB. The majority of new cases occurred in Asia (55%), followed by Africa (30%), the Eastern Mediterranea (7%), Europa (5%) and America (3%).

According to a report of WHO, "There are about 5% of previously treated or new TB patients being identified as MDR-TB infections in most countries. An estimation of 16% of 290 thousands of cases of MDR-TB may exist among notified TB patients

in 2010" [WHO, 2011]. The highest rates of the MDR-TB among new cases were discovered in former Soviet Union states and China, where a high percentage of patients fail to respond to standard drug therapy.

TB is the leading killer of HIV-infected individuals. In fact, it was estimated about 13% of TB infection cases reported among people living with HIV [WHO, 2011]. The rate of TB in areas with high HIV prevalence such as in Africa is much more than the rate of TB in low HIV prevalence areas. In developing countries, TB mainly affects young people who are the major economic labors of the society. Another serious fact is that TB is the main cause of death among women, especially in Africa. In 2010, there are 3.2 million women being identified as incident cases of TB, among them there are 0.32 million deaths [WHO, 2011].

1.6 The mechanisms of antibiotic resistance in mycobacteria

M. tuberculosis does not contain plasmid; they can develop resistance to antibiotic via accumulation of spontaneous chromosomal mutations. In general, resistance of mycobacteria to antibiotics can be classified as either intrinsic (natural) or acquired (chromosomal mutations). Resistance is usually provided by four different mechanisms:

1. modification of permeability of the cell wall
2. the activation of efflux pump system
3. drug degradations or modifications
4. drug target modifications

In particular, acquired drug resistance is mainly characterized by mutations in chromosomal genes, producing the selection of resistant strains during drug therapy.

1.6.1 Mycobacterial cell wall acts as a barrier against antibiotic penetration

Like the other free living bacteria, mycobacteria are covered by a cell wall. The cell wall not only keeps mycobacteria's shape but also protects them from harmful factors such as different osmotic pressure and physical or chemical compounds. Nevertheless, the chemical compositions of the cell wall and the assembly of the different macromolecules that make it up are modified during the growth and morphogenesis of cells. Mycobacteria have a special and complex cell wall with significantly low permeability. This characteristic serves as one of the major reasons of antibiotic resistance.

More than 60 % of the mycobacterial cell wall correspond to lipids. The structure of mycobacterial cell envelope usually consists of two lipid layers. The first one is a regular inner membrane, while second one is a layer mainly comprising of long-chain mycolic acids whose are covalently linked to peptidoglycan via arabinogalactan network (mAGP) [Minnikin, 1982]. When components of the cell wall are extracted, under the action of different detergents, beside soluble components such as proteins and free lipids, the mAGP complex remains insoluble.

Mycolic acids are long chains α-alkyl β-hydroxyl fatty acids, usually containing from 60 to 90 carbon atoms. They are strongly hydrophobic molecules forming the inner leaflet of the lipid bilayer of the cell wall. Lipoarabiomannan (LAM) and lipomannan (LM) are also forming the cell wall [Minnikin, 1982]. Mycolic acid layers are believed to have responsibility for the low permeability of mycobacteria. It works as the outer membrane of Gram-negative organisms and prevents the entry of hydrophilic compounds as antibiotics [Jarlier and Nikaido, 1994, Brennan and Nikaido, 1995]. However, mycobacteria cell wall is about a thousand times less permeable than Gram-negative bacteria such as *E. coli*.

It has been demonstrated that mycolic acid layers play an important role in forming an effective barrier against antibiotics. Liu and Nikaido [Liu and Nikaido, 1999] showed that the mycolate-deficient mutant failed to synthesize full-length mycolic acids and it increases susceptibility to hydrophobic compounds such as novobiocin,

rifampicin, erythromycin and crystal violet. The increase of the permeability was considered as one of the possible reasons for the increase of susceptibility to various hydrophobic compounds.

There are two different systems for biosyntheses of short chain and long chain fatty acids in mycobacteria. Indeed, the short chain fatty acids are synthesized by the fatty acid synthase I complex (FASI), while the long chain fatty acids are synthesized by the extension of the mercomycolate precursors from FASI by the FASII complex [Barry et al., 1998]. Cole et al. [Cole et al., 1998] showed that genes encoding FASII enzymes in *M. tuberculosis* are located in two loci: an operon having four genes: *fabD-acpM-kasA-kasB* and another *mabA-inhA* operon. These enzymes were tested as potential drug targets for TB.

The cell wall usually contains free lipids which are not covalently linked to peptidoglican-arabinogalactan such as asglycopeptidolipids or phthiocerol-dimycocerosates (DIMs/PDIMs) or the sulfolipids or the cord factor and so on [Minnikin, 1982]. These lipids have been shown to be important in maintaining an outer membrane like the structure of mycobacteria.

Free lipids play an important role in forming an effective barrier against antibiotics in *M. tuberculosis*. DIMs/PDIMs are produced by *M. tuberculosis* and a few other mycobacteria or most pathogenic in humans and animals. They are the main lipids of tubercle bacillus and play a role in the cell wall permeability [Camacho et al., 2001]. Many studies have implicated these molecules in the virulence of *M. tuberculosis*. Hydrophobic compounds can pass through the cell wall directly via the lipid membrane. Meanwhile the penetration of hydrophilic solutes is very low; they can diffuse via pore proteins-porins in the mycobacterial cell wall.

The porin of *M. chelonae* is the first one reported in mycobacteria. When the cell wall of *M. chelonae* is extracted by detergents, it is found to contain proteoliposomes, i.e. a channel-forming activity destroyed by protease. Subsequently, this channel-forming activity is purified and finally, it becomes a 59 kDa cell wall protein that allows the small and hydrophilic solutes to enter the cell [Trias et al., 1992]. The purified protein contains negative charges at its mouth and produces action-selective

channels. Nevertheless, unlike enterobacterial porin, the *M. chelonae* porin is a minor protein of the cell wall. Its amount is less than the amount of porin found in *E.coli*. Besides, it produces far lower permeability than that produced by the porin of *E. coli* [Trias et al., 1992]. These observations can be considered as an explanation of why the permeability of the *M. chelonae* cell wall to hydrophilic solutes is quite low.

In *M. tuberculosis* there is a molecule known as OmpATb which may play an important role in permeability to small hydrophilic solutes. OmpATb is a pore-forming protein and it appears to function as a porin. The OmpATb is required for adaptation of *M. tuberculosis* at low pH. The mutants of *M. tuberculosis* lacking of the *ompA* gene did not change their profile of antibiotics resistance [Raynaud et al., 2002] and up to now there have not been any evidence showing the participation of OmpA in the uptake of antibiotics.

MspA is the main porin of *M. smegmatis* and is responsible for the uptake of hydrophilic solutes. In *M. smegmatis*, there is a set of very similar porins known as Msp A, B, C and D. In wild type *M. smegmatis*, there are only expressions of *mspA* and *mspC*. When the *mspA* gene is deleted, the transcription of *mspB* and *mspD* is activated [Stephan et al., 2005]. It has been shown by Stephan et al. [Stephan et al., 2004] that porin pathway across the cell wall plays an important role in the sensitivity of both hydrophobic and hydrophilic antibiotics of *M. smegmatis*. MspA plays a major role in uptake of β-lactam antibiotics, moreover, the small and hydrophilic antibiotics such as, fluoroquinolones and norfloxacinalso cross the cell wall via Msp porin pathway. Other hydrophobic antibiotics such as chloramphenicol, erythromycin, novobiocin and rifampicin use porins to enter the cell as well [Danilchanka et al., 2008]. Mutants of *M. smegmatis* with a defected *mspA* gene increased resistance to the first line antibiotics such as rifampicin, ethambutol and isoniazid at high concentration as well as the second line antibiotics such as fluoroquinolones and cycloserine at concentration close to the MICs. It can also become multi-drug resistance [Stephan et al., 2004].

1.6.2 Efflux pump systems

Efflux pumps are one of the reasons of low-level intrinsic resistance in bacteria. The concentration of drug inside the cell depends on the balance of its influx and efflux [Viveiros et al., 2003]. Drug efflux pumps in bacteria were classified in several main families including the major facilitator superfamily (MFS), multidrug and toxic compounds extrusion (MATE), resistance-nodulation cell division (RND), small multidrug resistance (SMR) and the ATP binding cassette (ABC) [Li and Nikaido, 2004, Saier et al., 1998].

In mycobacteria, MFS, RND, SMR and ABC efflux pumps were known to involve in drug resistance. In the following subsections, we enumerate several mycobacterial drug efflux pumps which have been carefully studied.

MFS efflux pumps

Analysis of *M. tuberculosis* genome with bioinformatic tools helped identify 16 putative MFS efflux pumps [De Rossi et al., 2002]. However, not all of them were completely understood in their roles in drug resistance. *M. smegmatis* LfrA, was the first multidrug efflux pump reported in mycobacteria by [Takiff et al., 1996]. Overexpression of the *lfrA* gene confers resistance to fluoroquinones, acriflavine and other toxic compounds such as ethidiumbromide [Li et al., 2004, Sander et al., 2000]. The *lfrR*, i.e. an upstream of the *lfrA* gene, encodes a TetR-like regulator in which the *lfrR* gene acts as a repressor of *lfrA*. The deletion of the *lfrR* gene leads to strong expression of *lfrA* and causes the increase of high resistance to cationic dyes, fluoroquinolones and tetracycline. It also induces slightly resistance to INH [Li et al., 2004]. There is no homology of *lfrA* in *M. tuberculosis*, suggesting that there may be other efflux pumps contributed to the resistance of this mycobacterium to fluoroquinolones. Indeed, the Rv1634 efflux pump in *M. tuberculosis* confers resistance to various fluoroquinolones when it is over-expressed in *M. smegmatis* [De Rossi et al., 2002].

The Tet(V) is an efflux pump isolated from *M. smegmatis*. The increase of tetracycline MIC was observed when Tet(V) is over-expressed in *M. smegmatis*. However,

the analysis of the Tet(V) distribution showed that only *M. smegmatis* and *M. fortuitum* have this efflux pump [De Rossi et al., 1998a]. Other MFS efflux pumps were also identified in mycobacteria such as Tap and P55. Tap is an efflux pump of *M. fortuitium* and has a homology Rv1258c in *M. tuberculosis* and causes the resistance to aminoglicosides and tetracycline [Ainsa et al., 1998]. Moreover, the correlation between the transcription level of Rv1258c and drug resistance was determined in clinical *M. tuberculosis* isolates resistant to rifampicin and ofloxacin [Siddiqi et al., 2004]. The increase level of Rv1258c transcripts was observed when the isolates were grown in the presence of rifampicin and ofloxacin. However, both of these antibiotics were not shown to be substrates of Tap [Ainsa et al., 1998]. This suggests that efflux pumps may be stimulated in response to the presence of the corresponding drugs.

The P55 efflux pump in *M. bovis* and its *M. tuberculosis* homologue, known as Rv1410c, ia also associated with low-level resistance to aminoglycosides and tetracycline [Silva et al., 2001]. Recently, it has been shown that mutants lacking of P55 in *M. bovis* BCG increased susceptibility to various toxic compounds including rifampicin and clofazimine [Ramon-Garcia et al., 2009].

The *epfA* gene encodes a putative efflux pump EpfA in *M. tuberculosis* H37Rv. The secondary structure of this efflux pump is similar to that of members of transporter family QacA. To the best of our knowledge the association between EpfA and drug resistance in *M. tuberculosis* has not been well understood yet. Nevertheless, the deletion of *epfA* homologue in *M. smegmatis* causes an increase of susceptibility to ethidium bromide, gentamicin, FQ and acriflavine [De Rossi et al., 2006].

SMR family drug transporters

Mmr encoded by the *mmr* gene is a member of the SMR family, which was identified in *M. tuberculosis*. Other *mmr*-like genes exist in other Mycobacterium species including: *M. simiae*, *M. gordonae*, *M. marinum*, *M. smegmatis* and *M. bovis* [De Rossi et al., 1998b]. The expression of the *mmr* gene in *M. tuberculosis* or in *M. smegmatis* results in resistance to tetraphenyl phosphonium, ethidiumbromide, erythromycin, safranin O and pyronin Y [De Rossi et al., 1998b]. Moreover, mutants with the deletion of the

mmr homologue in *M. smegmatis* increase susceptibility to cationic dyes and flouroquinolones.

RND drug transporters

The genome sequence of *M. tuberculosis* consists of 13 putative transporters predicted to belong to RND family [Cole et al., 1998]. The MmpL (mycobacterial membrane proteins, large) appears to be confined to mycobacteria [Pasca et al., 2005]; they share sequence and structure similarities to each other. The *mmpL7* gene of *M. tuberculosis* confers high level resistance to INH (MIC is 32 times higher than wild type) when it is over expressed in *M. smegmatis*. This fact indicates that the MmpL7 protein actively pumps out INH in *M. smegmatis* [Pasca et al., 2005].

ABC drug transporters

There are at least 37 ABC transporters completely and incompletely identified in *M. tuberculosis* [Braibant et al., 2000]. Only a few of them has been characterized and determined to play a role in drug resistance in *M. tuberculosis*. The genome sequencing and analyzing of *M. tuberculosis* show that *M. tuberculosis* has the doxorubicin-resistance operon, i.e. *drrAB* [Cole et al., 1998]. The expression of the *drrAB* genes in *M. smegmatis* confers resistance to antibiotics such as tetracycline, erythromycin, ethambutol, norfloxacin, streptomycin and chloramphenicol. This resistant phenotype can be reversed by treatments with verapamil or reserpine which are known as efflux pump inhibitors [Choudhuri et al., 2002].

Phosphate specific transporter (Pst) has been reported in many bacteria including *M. tuberculosis*. The high level of transcription [Banerjee et al., 1998] and chromosomal amplification [Banerjee et al., 2000] of the *pstB* gene encoding PstB was observed in the *M. smegmatis* ciprofloxacin resistant mutant generated in a laboratory. The active efflux pump plays a major role in resistance to ciprofloxacin of this strain [Bhatt et al., 2000]. An ABC transporter encoded by the Rv2686c-Rv2687c-Rv2688c operon of *M. tuberculosis* plays an important role in fluoroquinolones efflux when it is produced from a multi-copy plasmid. Over-expression of this operon in *M. smeg-*

matis confers resistance to ciprofloxacin and norfloxacin. Pump inhibitors reserpine, verapamil and carbonyl cyanide m-chlorophenylhidrazole (CCCP) reduce the level of resistance [Pasca et al., 2004].

1.6.3 Drug tolerance

The tolerance of *M. tuberculosis* to several commonly used antibiotics limits the chemotherapy. In many cases, it is considered as the root of TB treatment failure. Morris et al. [Morris et al., 2005] described a mycobacterial system interconnecting with resistance to antibiotics penetrating the cell wall; it enables mycobacteria to tolerate to antibiotics which inhibit cytoplasmic targets. This system depends on *whiB7* which is a gene encoding a WhiB7 transcriptional regulator. WhiB7 belongs to a family of regulator proteins WhiB which is restricted to the Actinomycetes [Soliveri et al., 2000]. In Steptomyces coelicolor there are 14 genes belonging to the *whiB* family, among them *whiB7* plays a role in multidrug resistance phenotype. The replacement of *whiB7* in *M. tuberculosis*, *M. bovis* BCG or in *M. smegmatis* (Nguyen et al., unpublished manuscript) causes the sensitivity to variety of antibiotics including macrolides, a lincosamide, and an aminoglycoside [Morris et al., 2005, Nguyen and Thompson, 2006].

Moreover, the expression of *whiB7* is significantly induced by minimal inhibition concentration of erythromycin, tetracycline and high concentration of streptomycin. It also controls the expression of a regulon that contains at least eight genetic loci including two well documented antibiotic resistant genes *tap* and *erm* [Morris et al., 2005]. Similar to the other WhiB proteins, WhiB7 seems to work together with the primary sigma factor (SigA) in control of its regulon. Nevertheless, genome of *M. tuberculosis* encodes 12 alternative sigma factors, among them there is SigF being antibiotic-inducible and taking part in intrinsic multidrug resistance.

The *iniBAC* operon is strongly induced when *M. bovis* BCG or *M. tuberculosis* are treated with ethambutol or INH. This operon confers multidrug tolerance via an associated pump-like activity [Colangeli et al., 2005]. Over-expression of the *M. tuberculosis iniA*gene in the *M. bovis* BCG under exposure to either INH or etham-

butol may result in tolerance to these antibiotics. *M. tuberculosis* mutants with *iniA* deletion increase susceptibility to INH [Colangeli et al., 2005].

Lsr2 is a small histone-like protein with broad down-regulatory and up-regulatory activities in *M. smegmatis*. In *M. tuberculosis* it controls the *iniBAC* operon and the other antibiotic-induced genes including the *efpA* gene. This protein regulates genes by modifying the DNA shape; it introduces a small number of coils into the DNA structure. Therefore, Lsr2 is broadly considered as one of the major regulators of antibiotic-induced responses in mycobacteria [Colangeli et al., 2007].

1.6.4 Molecular mechanisms of resistance to the first and the second line anti-TB drugs

Isoniazid

Isoniazid (INH) is one of the first-line antibiotics in the treatments of tuberculosis. It was first reported as anti-tuberculosis in 1952. Isoniazid has a very simple structure consisting of a pyridine ring and a hydrazide group. These two components are believed to have an essential role in the high activity against *Mycobacterium tuberculosis*.

Isoniazid enters the mycobacterial cell through passive diffusion [Bardou et al., 1998]. It is a prodrug which must be activated mainly by the mycobacterial catalase-peroxidase enzyme KatG encoded by the *katG* gene [Zhang et al., 1992]). In accordance with its peroxidase activity, KatG activates INH by peroxidation to produce intracellular reactive INH species. It is known that INH inhibits the synthesis of both mycolic and nucleic acids. However, the mechanisms behind these processes are still unclear and required more deep investigations.

In the presence of NADH (NAD^+ or $NAD^{\cdot}$ radical) reactive INH intermediates lead to the formation of INH-NAD adducts which are powerful inhibitors of InhA. InhA is an enoyl acyl carrier protein reductase [Banerjee et al., 1994, Dessen et al., 1995] known as an important enzyme involving in mycolic acid biosynthesis. Moreover, INH reacts with $NADP^+$ to form an INH-NADP abduct strongly inhibiting MabA,

an NADPH dependent β-ketoacyl-ACP reductase which also plays an important role in mycolic acid biosynthesis [Ducasse-Cabanot et al., 2004]. Thus, INH-NAD and INH-NADP adducts can inhibit different steps of cell wall lipid synthesis via inactivation of important enzymes of this process including InhA and MabA, Furthermore, it has been shown that the INH-NADP adduct binds *M. tuberculosis* dihydrofolatereductase (DHFR) and blocks DHFR activities [Argyrou et al., 2006]. DHFR is central in nucleic acid biosynthesis to make the nucleotide pools, thus its inhibition leads to the prevention of the nucleic acid synthesis. In addition to that, another enzyme target of INH is a complex of an acyl carrier protein (AcpM) and a β-ketoacyl-ACP synthase (KasA), an enzyme taking part in mycolic acid synthesis.

Mutations in *katG* are the main mechanisms of INH resistance. Indeed, mutants in *katG* have high level resistance to INH [Winder, 1982]. About 50% of INH resistant clinical isolates of *M. tuberculosis* have deletions or missense mutations in the *katG* gene. The Ser315Thr mutation is reported as the most frequent mutation among isoniazid resistant mutations. The enzyme KatG with Ser315Thr mutation losses its ability to activate INH, but it still remains about 50% of its catalase-peroxidase activity [Rouse et al., 1996].

Resistance to INH may also be the results of other mutations in the promoter regions of the *mabA-inhA* operon which leads to over expression of InhA or mutations in the *inhA* gene causing the decrease of the InhA affinity to the INH-NAD adducts [Rozwarski et al., 1998, Banerjee et al., 1994]. These mutations usually confer low-level resistance. INH resistant mycobacteria containing mutation in the *inhA* gene may have mutation in *katG*, they confers high level of resistance [Heym et al., 1995].

In addition to the aforementioned mutations, the mutations in the genes *kasA*, *acpM* in clinical isolates exhibit low-level resistance to INH [Zhang and Telenti, 2000]. However, the role of KasA mutation in resistance to INH is still unclear. The reason is that similar mutations are also found in INH-susceptible strains;in the case of resistance to INH, mutations were also determined in the *katG* or the *inhA* genes [Lee et al., 1999, Piatek et al., 2000].

Mutations in the promoter region of *ahpC*, encoding an alkylhydroperosidereduc-

tase, may be another reason of INH resistance. These mutations cause over expression of the AhpC enzyme and do not confer high-level resistance. The INH resistant strains with these kinds of mutants also possess *katG* mutations [Zhang and Telenti, 2000, Ramaswamy and Musser, 1998, Sherman et al., 1996]. The over expression of AhpC was observed as a compensation for the lack of catalase-peroxidase in these mycobacterial strains [Wilson et al., 1998].

In *M. smegmatis*, mutations in *ndh* reducing the activity of NADH dehydrogenase cause resistance to INH and ethionamide [Miesel et al., 1998]. In *M. tuberculosis*, INH resistant strains with mutations in *ndh* may also have mutations in other genes such as *inhA* or *katG*.

Recently, down regulation of *katG* expression has been determined to have a relation with INH resistance. Three mutations in *furA-katG* intergenic region substitution were found in INH resistant clinical isolates of *M. tuberculosis*. Mutations in this region decrease the *katG* expression, thus, confer resistance to INH [Ando et al., 2011]. Furthermore, mutations in the intergenic region of *oxyR-aphC* reduce the level of expression of *inhA* and also have been associated with INH resistance. These mutations have been reported in 4.8% to 24.2% of INH resistant *M. tuberculosis* isolates [Dalla Costa et al., 2009].

In addition, [Sholto-Douglas-Vernon et al., 2005] showed that INH is activated by the human N-Acetyl transferase (NAT2), as well as by the *M. tuberculosis* NAT enzyme [Upton et al., 2001]. When both *nat* genes of *M. tuberculosis* and *M. smegmatis* were expressed in *M. smegmatis*, an increase of INH resistance was observed [Payton et al., 1999]. Moreover, the deletion of *nat* in *M. smegmatis* causes the increasing sensitivity to INH [Payton et al., 2001].

Rifampicin

Rifampicin (rifampin) is a bacterial antibiotic drug of the group rifamycin. Rifampicin was discovered in 1963 and introduced for TB chemotherapy in 1971 as a major addition to the treatments methods of tuberculosis and inactive meningitis, along with isoniazid, ethambutol, pyrazinamide and streptomycin. Currently, rifampicin

is one of the most important anti-TB drugs in the treatments of TB in short-term chemotherapy [Mitchison, 1992].

According to [Cole, 1994, Rastogi and David, 1993], rifampicin inhibits bacteria RNA-polymerase activity; it binds β-subunit of RNA-polymerase and forms a stable drug-enzyme complex . Therefore, the transcription of RNA from DNA template is prevented. One of the most important characteristics of rifampicin is its great activity against actively growing and slow metabolizing (non-growing) bacilli. Recently, resistance to rifampicin is increasing rapidly as a result of the widespread of inappropriate antibiotic usage.

Resistant mutant do not form complex between enzyme and rifampicin. Most *M. tuberculosis* rifampicin resistance strains arise because of mutations in the *rpoB* gene, which encodes the RNA polymerase β-subunit [Wehrli et al., 1968]. It was estimated that around 95% of rifampicin resistant *M. tuberculosis* strains had mutations located in the 81-bp region of the *rpoB* gene (codons 507 to 533). This region is also known as the rifampicin resistance-determining region (RRDR). In most of the studies, the bacterial strains with high resistance levels tend to harbor mutations at codons 526 and 531 (about 60% of all mutations). Besides, the other additional mutations have been mapped to positions 511, 512, 513, 516, and 533. These mutations do not correlate with the increasing levels of resistance [Cummings and Segal, 2004, Morlock et al., 2000]. Rifampicin resistant tuberculosis is often observed in combination with resistance to the other drugs, leading to long-term treatments and remarkably worse chemo-therapeutic outcomes. About 90% of rifampicin resistant clinical isolates are also isoniazid resistant, thus, rifampicin resistance is a positive indicator of multi-drug or extensive-drug resistance.

However, rifampicin resistant strains of *M. tuberculosis* with no mutations in 81-bp region of the *rpoB* gene have been reported [Ohno et al., 1996]. Furthermore, most of rifampicin resistant clinical isolates of *M. avium* and *M. intracellulare* did not have any mutations in the *rpoB* gene [Guerrero et al., 1994], in *M. smegmatis*, *rpoB* mutations have not yet been identifiable [Hetherington et al., 1995]. These data suggest that there are mechanisms of rifampicin resistance being not fully addressed

in the literature. To the best of our knowledge, the permeation and transport of antibiotics into mycobacteria are still not yet well understood. Although there are only a few studies on this topic, rifampicin, i.e. a hydrophobic antibiotic, is believed to enter the mycobacteria cell through direct diffusion across the mycobacterial cell wall. Most part of natural resistance of mycobacteria to rifampicin can be attributed to a permeability of lipid-rich through mycobacterial cell wall [Hui et al., 1977].

Pyrazinamide

Pyrazinamide is an important first line anti-TB drug; it was discovered in 1952 and was not used extensively in TB treatment until 1980s. Utilization of this drug helps treatment duration to be reduced from 9-12 months to 6 months. The drug is most active against TB at acid pH ($pH < 6$) [McDermott and Tompsett, 1954]. The activity of pyrazinamide increases under low oxygen or anaerobic conditions [Wade and Zhang, 2004].

Pyrazinamide is a prodrug entering *M. tuberculosis* cells through passive diffusion. It must be activated to have its active form, i.e. pyrazinoic acid by the pyrazinamidase/nicotinamidase enzyme (PZase) which is encoded by the *pncA* gene in *M. tuberculosis* [Scorpio and Zhang, 1996]. Acid pH condition stimulates the formation of the protonated pyrazinoic acid which then crosses the membrane and causes increasing accumulation of pyrazinoic acid anions and protons in the cell [Zhang et al., 1999]. The accumulation of protons is a reason of the disruption of membrane potential which is a main contributor of the proton motive force. The disruption of membrane potential leads to the inhibition of the membrane transport. These facts indicate that pyrazinoic acid points to the membrane energy metabolism [Zhang et al., 2003]. Pyrazinoic acid and its n-propyl ester were thought to inhibit enzyme fatty acid synthesis Fas-I [Zimhony et al., 2000], but its validity is still questioning [Boshoff et al., 2002].

According to [Scorpio and Zhang, 1996, Scorpio et al., 1997, Cheng et al., 2000] mutations in *pncA* are the major mechanisms of pyrazinamide resistance. Indeed, most of mutations occur in the *pncA* gene and in its putative promoter regions [Scorpio et al., 1997, Juréen et al., 2008]. These mutants results in the loss or reduc-

ing activity of PZase [Scorpio et al., 1997]. Between 72-97% of pyrazinamide resistant *M. tuberculosis* strains have mutations in *pncA* [Scorpio et al., 1997, Louw et al., 2006, Portugal et al., 2004]. However, some resistant strains do not have mutations in *pncA*. It has been suggested that pyrazinamide resistance may be provided by mutations in an unknown *pncA* regulator gene. Furthermore, another type of similar resistant strains without *pncA* mutations has low-level resistance and retains PZase activity; the mechanisms of such pyrazinamide resistance remain unidentified [Sreevatsan et al., 1997b].

Pyrazinamide is highly active against *M. tuberculosis*, but it has a little or no activity against other mycobacteria such as *M. bovis*. The reason may be due the difference between the *pncA* genes in many species of mycobacteria [Sun and Zhang, 1999]. Scorpio et al. [Scorpio and Zhang, 1996] shows that strains of *M. bovis* are naturally resistant to pyrazinamide and do not have PZase, these characteristics are used to differentiate *M. bovis* from *M. tuberculosis*.

Ethambutol

Ethambutol is a first line drug that is usually used in combination with INH, rifampicin, pyrazinamide and streptomycin to avoid the spread of drug resistance in TB. It was first discovered in 1962 and up to date it has been using as a part of a standard treatment regimen for TB. Ethambutol is active against growing bacilli and almost has no effect on non-replicating bacilli. It intervenes in biosynthesis of cell wall agabinogalactan [Takayama and Kilburn, 1989]. The target of ethambutol was identified to be arabinosyl transferase encoded by *embB*, an enzyme taken part in the synthesis of arabinogalactan, in *M. tuberculosis* and in *M. bovis*. In *M. tuberculosis*, *embB* belongs to an operon together with *embA* and *embC* in the order *embCAB*. They share more than 65% of amino acid identity with each other and are predicted to encode transmembrane proteins [Telenti et al., 1997, Belanger et al., 1996].

Mutations in the *embCAB* operon, usually in *embB* and occasionally in *embC*, are the main mechanisms of resistance to ethambutol [Telenti et al., 1997]. According to [Sreevatsan et al., 1997a], most of ethambutol resistant isolates in *M. tuberculosis*

have mutations in *embB* . Mutations in *embB* codon 306 happen most frequently in up to 20% of ethambutol-susceptible isolates [Lee et al., 2004, Ahmad et al., 2007]. Some studies have suggested that *embB306* mutations did not cause the classical ethambutol resistance. Instead, they were found to be associated with resistance to other antibiotics and to multidrug resistance [Hazbon et al., 2005, Shi et al., 2007, Perdigao et al., 2009]. Analysis of individual mutations causing different amino acid substitutions showed that mutations producing certain amino acid changes caused ethambutol resistance, while other amino acid substitutions had little or no effect on ethambutol resistance. Mutations at *embB306* also appeared to be necessary, but not sufficient, to produce high-level EMB resistance in the clinical strains. Therefore, these mutations must contribute indirectly to high-level resistance, probably, via interaction with mutations in other genes [Safi et al., 2008].

However, there is a proportion about 35% of ethambutol resistant strains observed without mutations in *embB*. This number is significant suggesting that there may be other unknown mechanisms of resistance to ethambutol [Alcaide et al., 1997].

Aminoglycosides: streptomycin, kanamycin, amikacin and capreomycin

Steptomycin was first isolated in 1943 and was the first antibiotic that could be used for TB treatment. It is an alternative first line anti-TB drug recommended by WHO [Cooksey et al., 1996]. Therefore, streptomycin is usually used in the treatment regimen of TB in combination with four other drugs including INH, rifampicin, pyrazinamide and ethambutol.

Streptomycin is an inhibitor of protein synthesis. Indeed, it binds to the 30S subunit of ribosome, leading to misreading of mRNA during translation [Davies et al., 1965]. Streptomycin interacts with the 16S rRNA encoded by the *rrs* gene and S12 ribosomal protein encoded by the *rpsL* gene [Finken et al., 1993]. Mutations in the *rrs* gene and the *rpsL* gene are the major original causes of resistance to streptomycin. About 50% of streptomycin resistant strains have mutation in the *rpsL* gene. Among them mutations causing a change from Lys to Arg in codon 43 and 88 are the most common and result in high-level of resistance. There are about 20% streptomycin resistant isolates

harbor mutations in the *rrs* gene. These mutations were found mainly in the loops of the 16S rRNA, in two regions around nucleotides 530 and 915 [Finken et al., 1993, Nair et al., 1993]. However, low-level streptomycin resistant strains without mutations in both of the *rrs* genes and the *rpsL* genes were reported. Hence, there might be an alternative mechanism for streptomycin resistance [Cooksey et al., 1996]. Recently, mutations in the *gidB* encoded a conserved 7-methylguanosine methyltransferase specifically for the 16S rRNA have been discovered as a main reason of low-level resistance to streptomycin [Okamoto et al., 2007, Spies et al., 2008].

Kanamycin and amikacin also inhibit protein synthesis by modification of ribosomal structures at the 16S rRNA. High-level resistance to kanamycin and amikacin is provided by mutations at codon 1400 of the *rrs* gene [Alangaden et al., 1998, Suzuki et al., 1998].

Capreomycin is a peptide antibiotic. Mutations in the *tlyA* gene are associated with resistance to capreomycin. This gene encodes an rRNA methyltransferase. The activity of this enzyme is lost when mutations occur in the *tlyA* [Maus et al., 2005].

Cross-resistance maybe found between kanamycin, amikacin and capreomycin [Winder, 1982]. The A1401G mutation in the *rrs* gene causes resistance to all the three antibiotics. Mutant strains being resistant to kanamicin and capreomycin could have C1402T or G1484T mutation in the *rrs* gene. Multiple mutations may also happen in the *rrs* gene in one strain causing cross-resistance among these antibiotics [Maus et al., 2005].

Fluoroquinolones

Fluoroquinolones are broad-spectrum antibiotics being very important in treatments of serious bacterial infections. These antibiotics are currently used as second-line drug in TB treatments. The targets of fluoroquinolones in most bacterial species are DNA gyrase (topoisomerase II) and topoisomerase IV. DNA topoimesomerases are responsible for maintaining chromosomes in an appropriate topological structure [Drlica and Malik, 2003]. *M. tuberculosis* only have DNA gyrase which is a tetrameric protein containing A and B subunits encoded by the *gyrA* and the *gyrB* genes re-

spectively [Wang, 1996, Takiff et al., 1994]. Fluoroquinolone resistance is a result of mutations in the putative fluoroquinolone binding to a region in *gyrA* or in *gyrB*. It is a conserved region, i.e. the quinolone resistant determining region (QRDR) of *gyrA* (320 pb) and *gyrB* (375 pb) which was determined to play the most important role in resistance to fluoroquinolones [Takiff et al., 1994]. Among mutations in *gyrA* of *M. tuberculosis*, the most frequently mutated positions were identified at codons 90 and 94. Nevertheless, other mutations at codons 74, 88 and 99 have also been reported [Chen et al., 2004, Sun et al., 2008]. Mutation at codon 95 contains a polymorphism and it is not involved in quinolone resistance [Sreevatsan et al., 1997a]. Mutations in *gyrB* seem to be very rare.

There are other mechanisms of fluoroquinolone resistance. The contribution of efflux pumps in resistance to these antibiotics was mentioned in the efflux pump part of this thesis. Recently, resistance to quinolones mediated by MfpA was determined. MfpA is a member of the pentapeptide repeat family of proteins from *M. tuberculosis*. Its expression causes resistance to ciprofloxacin and sparfloxacin. This protein binds to DNA gyrase and inhibits its activity. The MfpA with three-dimensional structure contains a fold named as the right-handed quadrilateral -helix, which displays size, shape and electrostatic similarity to B-form DNA. It was suggested that MfpA competes with B-from DNA for the gyrase surface. MfpA binding to DNA-gyrase forbids the formation of DNA gyrase-DNA complex which is the target of fluoroquinolonee. Therefore it prevents the lethal interaction of the drugs with DNA gyrase and provides a molecular explanation for the antibiotic resistance mechanism [Hegde et al., 2005]. In addition, the mutations in the *gyr* genes confer high-level resistance to fluoroquinolones, while the alternative mechanisms usually relate to low-level of resistance [Chen et al., 2004]. However, the combination of *gyr* mutations and alternative mechanisms results in a considerable level of resistance to fluoroquinolones.

Ethionamide

Ethionamide is an important drug usually used for treatments of MDR-TB. Similar to isoniazid, ethionamide is a prodrug that needs to be activated by a mono-oxygenase

EthA encoded by the *ethA* genes [DeBarber et al., 2000]. EthA is a flavin adenosine dinucleotide. It activates ethionamide through oxidation to active intermediates which subsequently form adducts with NAD. The ethionamide-NAD adduct inhibits the same target as the INH, particularly, the InhA of the mycolic acid synthesis pathway [Banerjee et al., 1994].

Mutations in the *ethA* gene and the *inhA* gene result in the resistance to ethionamide [Hazbon et al., 2006]. Moreover, mutations in *inhA* may alter the InhA target or mutations in the promoter region of *inhA* causing the over-expression of *inhA* and confer cross-resistance to ethionamide and isoniazid [Vilcheze et al., 2006]. In addition to that, cross-resistance to these drugs is also a result of mutation in *ndh* which increases the intracellular concentration of NADH [Vilcheze et al., 2005].

Moreover, the expression of *ethA* is negatively regulated by the *ethR* gene encoding of the EthR protein [Baulard et al., 2000, DeBarber et al., 2000]. EthR belongs to the TetR/CamR family of transcriptional regulators; it binds to the *ethA* operator, 5 to 16 nucleotides upstream from the *ethA* gene start codon [Aramaki et al., 1995, Engohang-Ndong et al., 2004]. The over expression of the *ethR* genes causes repression of *ethA* expression, thus, it results in ethionamide resistance [Baulard et al., 2000, DeBarber et al., 2000].

Chapter 2

Research Objectives

In this thesis, we studied mutagenesis effect of antibiotics and mechanisms of antibiotic resistance of bacteria. Two model organisms were used: *Escherichia coli* and *Mycobaterium smegmatis*.

2.1 Mutagenic effect of antibiotics in *E. coli*

Low concentrations of some antibiotics have been reported to stimulate mutagenesis and recombination, whose may facilitate adaptation of bacteria to different types of stresses, including antibiotic pressure. However, the mutagenic effects of most of the currently used antibiotics remains untested. In this part, we would like to study the roles of antibiotics in the stimulation of mutagenesis of *E. coli* via SOS response. Indeed the effect of *recA* inactivation on mutagenesis was studied under the treatments of variety antibiotics with different concentrations around their MIC. In order to achieve the aforementioned goals our studies are planned as follows:

- To study the mutagenic effect of difference antibiotics on the *E. coli* E12 strains by evaluating mutant frequency of bacteria in rifampicin and fosfomycin resistance.

- To study the effect of antibiotics on the induction of *recA* transcription

- To study the effect of antibiotics on mutant frequency in the mutant lacking of the *recA* gene.
- To study the effect of antibiotics on cell morphology.

2.2 Antibiotic resistance in *M. smegmatis*

Antibiotic resistances in mycobacteria is one of the most emergency issues in the world. In this thesis, we would like to analyze the possible resistant mechanisms in the model organism *M. smegmatis*. It has been well-known that mutation in *rpoB* is one of the main mechanisms of rifampicin resistance. However, there are alternative mechanisms of rifampicin resistant which are not provided by mutations in *rpoB*. Therefore, in this study we aim at characterizing those mechanisms in *M. smegmatis*. The plan of our study is as follows:

- To generate an insertion mutant library of *M. smegmatis* by tranposon mutagenesis using ϕMycoMarT7.
- to screen the mutant library for mutants that may be resistant to rifampicin.
- To characterize the rifampicin resistant candidates with disruptions in the *trkA* and *ich* genes, focusing in the following characteristics:
  - Antibiotic resistant profile
  - Growth properties under different conditions including at normal conditions and at acidic pH conditions
  - The membrane permeability (via evaluating the membrane potential)

Chapter 3

Materials and Methods

3.1 Bacterial strains and plasmids

Bacterial strains used in this thesis are: *Mycobacterium smegmatis* wild-type strain mc^2 155. The *Escherichia coli* K-12 strains used were: MG1655 (F$^-$, lambda$^-$, rph-1, wild-type K-12 strain) ME12 (MG1655 *lacZDC-lacZDN-yfp*) and an ME12 *recA* :: *kan* derivative (ME12 recA938::Tn5 (Kn^R)). *Escherichia coli* DH5α (F$^-$, endA1, hsdR17 (r$^-$, m$^+$), supE44, thi1, recA1, gyrA, relA1, D(lacZYA, argF)U169, 80 [D lacZM15]).

Plasmids are listed in the following table:

Plasmid	**Description**	**Source or reference**
p2NIL	Gene manipulation vector, Kn^R	[Parish and Stoker, 2000]
p2NIL-Δ*trkA*	p2NIL containing an in-frame deletion of the trkA gene	This study
p2NIL-Δ*ich*	p2NIL containing an in-frame deletion of the ich gene	This study
pGOAL19 HygR	P*Ag85*-lacZ P*hsp60*-sacB^S PacI cassette vector, Amp^R	[Parish and Stoker, 2000]
pVV16	Mycobacteria expression vector with Hyg^R, Kn^R and Phsp60	[Schulbach et al., 2001]
pvv16-*trkA*	pvv16 containing the *trkA* gene	This study
pvv16-*ich*	pvv16 containing the *ich* gene	This study
pSC101-PrecA::GFP	GFP transcriptional fusion after the promoter of the *recA* gene with Kn^R	[Ronen et al., 2002]

Table 3.1: Plasmids

3.2 Bacterial media and growth conditions

LB (Luria-Bertani) broth (USB Corporation): 20g was added to 1L purified water. For LB agar 15g of agar was added to the liquid media and sterilized by autoclaving.

Difco Middlebrook 7H9 broth (BD biosciences): 4.7g was added to 900 ml purified water and was sterilized by autoclaving, 100 ml Middlebrook ADC enrichment (BBL) and 0.05% Tween80 was added before using.

Difco Middlebrook 7H10 agar (BD biosciences): 19g was added to 900 ml purified water and sterilized by autoclaving, add 100ml Middlebrook ADC enrichment (BBL) and 0.05% Tween80 was added before using.

Media supplements: all media supplements were obtained from Sigma. They are listed with their stock concentrations as following: hygromycin B at 100 mg/ml, kanamycin at 50 mg/ml, 5-bromo-4chloro-3-indolyl-b-D-galactoside (X-gal) at 20 mg/ml in DMSO, sucrose at 40mM.

M. smegmatis wild-type strain mc^2 155 and its mutant derivatives were grown at 37^{o}C in Middlebrook 7H9 broth or Middlebrook 7H10 agar. For strain selection, media were supplemented with 25 μg/ml kanamycin or 50 μg/ml hygromycin B or 40 mg/ml XGAL or 10 mM sucrose, when necessary. *E. coli* DH5a strain was cultured at 37^{o}C in LB medium containing 50 μg/ml kanamycin or 100 μg/ml hygromycin, when appropriate.

Long-term storage of bacterial strains was carried out by adding 0.2 ml of 80% v/v sterile glycerol to 1 ml aliquots of an overnight culture and stored at -80^{o}C.

3.3 General techniques of bacterial genetics

The methods for preparation of competent or electro-competent cells, transformation by heat-shock or electroporation, transduction with phage and so on follow the instruction of the techniques described in the literature by Sambrook [Sambrook et al., 1989], Miller [Miller, 1992], Parish and Brown [Parish and Brown, 2011].

3.4 DNA techniques

All of the techniques such as restriction digestion of DNA, ligation of DNA fragments, agarose gel electrophoresis and isolation of *M. smegmatis* genomic DNA were designed based on the protocols proposed by Sambrook [Sambrook et al., 1989] and Parish and Brown [Parish and Brown, 2011]. Besides, the plasmids of *E.coli* were purified using the Qiagen QiAprep Spin Miniprep kit.

The specific amplification of DNA regions was done using the polymerase chain reaction. PCR reactions were performed in 50μL volumes containing 1.25U AmpliTaq Gold DNA polymerase (Roche), 0.2mM each dNTP, 1.5mM of MgCl2, 0.5 μM each primer, 20ng of DNA template and 1 x PCR buffer.

3.5 Colony PCR

The reaction was carried out in 50μL volumes and the same PCR cycle was also maintained. The colony of *E.coli* was re-suspended in the PCR mix and the PCR performed on it. In the case of colony PCR of *M. smegmatis*, the colony was re-suspended in 50ml sterile water in an eppendorf. Later on, the mix was being incubated at 90^{o}C for 15 minutes before being centrifuged and adding 3μml of the supernatant to the PCR mix as a template.

The purification of PCR products and restriction digests was carried out using the Qiagen QiAquick PCR Purification Kit in accordance with the manufacturer's instructions. For the purification of DNA from an agarose gel slice the Qiagen QiAquick Gel Extraction kit was used.

3.6 DNA sequencing

The sequence of plasmids and PCR products were carried out by the sequencing services of the company Secugen[1] using the modified dideoxy-chain termination method. The oligos used for reactions are shown in Table 3.2.

[1]http://www.secugen.es

3.7 Oligos used in this study

All oligos were synthesized by Sigma-Aldrich and are listed in the following table (underlined bases denote restriction sites):

Name	**Sequence (5'↦3')**	**Description**
DeltrkA5'F	CCCGCTGCAGGCCGTGTTGGCGGCCAGACA	Fwd primer to amplify a fragment up-stream of *trkA* from *M. smegmatis*
DeltrkA5'R	GTCCAAGCTTTCGACCGCAGCGCG	Rev primer to amplify a fragment up-stream of *trkA* from *M. smegmatis*
DeltrkA3'F	CCTCAAGCTTCCTGGATTCCGAATGAGCCG	Fwd primer to amplify a fragment down-stream of *trkA* from *M. smegmatis*
DeltrkA3'R	GGTGGGTACCGACGACGCGGGCTGGCGCGA	Rev primer to amplify a fragment down-stream of *trkA* from *M. smegmatis*
Delich5'F	GACAAAGCTTCGGCCACCGCGCGTGCGGTT	Fwd primer to amplify a fragment up-stream of ich from *M. smegmatis*
Delich5'R	GCGAGGTACCATTCGAGTCGATGGCGGCCA	Rev primer to amplify a fragment up-stream of ich from *M. smegmatis*
Delich3'F	GGCCGGTACCCGGCTCCTGTACATCCGCAG	Fwd primer to amplify a fragment down-stream of ich from *M. smegmatis*
Delich3'R	CCAAGCGCGGCCGCCCTTATCCCGAGAAT GGGCCAC	Rev primer to amplify a fragment down-stream of ich from *M. smegmatis*
trkAF	GGCCGCCATATGACCAGTCGGCGCGCTGCG	Fwd primer to amplify *trkA* from *M. smegmatis*
trkAR	CCGCCTGCAGTCATTCGGAATCCAGGTCGT	Rev primer to amplify *trkA* from *M. smegmatis*
ichF	GGATGACATATGGCTAAAGGCAGGTTACGG	Fwd primer to amplify *ich* from *M. smegmatis*
ichR	CGGTAAGCTTTCATCGTTCGGCGTCCGCAC	Rev primer to amplify *ich* from *M. smegmatis*
MycoMar specific primer	CCCGAAAAGTGCCACCTAAATTGTAAGCG	Primer for sequencing to identify the transposon insertion site
seqichF	GGTGCGGTCATGGTTACATTG	Fwd primer for sequencing checked the *ich* knockout mutant
seqichR	GACAGCAGCGGGACGTTAC	Rev primer for sequencing checked the *ich* knockout mutant
seqtrkA	CATCCAGTCTATTAATTGTTGCCG	Fwd primer for sequencing checked the trkA knockout mutant
seqtrkAR	CTGCGCAGGGCTTTATTGATTCCA	Rev primer for sequencing checked the *trkA* knockout mutant

Table 3.2: Oligonucleotides

3.8 Mutagenesis experiments

Mutant frequencies were obtained in the same way as described in the literature [Lopez et al., 2007, Elez et al., 2007]. Briefly, for mutant frequency, 2 ml aliquots of exponentially growing cells (10^8 cells/mL) were incubated with different concentrations of antibiotic for 4 hours at 37^oC with shaking (250 rpm). One ml of these cultures was centrifuged for 10 minutes at 6000 rpm in a minifuge. The pellet was resuspended in 2 ml of fresh LB medium and incubated overnight at 37°C with

shaking. This step is necessary to resolve the filaments formed after treatments with some antibiotics, such as ciprofloxacin, ceftazidime, trimethoprim and trimethoprim/sulfamethoxazole. Resolution of filaments was verified by direct observation of samples from the different cultures under the microscope. Only cultures with a proportion of filaments of less than 5% of total cells were plated. Viable cells were determined by plating appropriate dilutions onto LB agar plates. Mutant frequencies were obtained as the number of colonies growing on rifampicin (100 μg/ml) or fosfomycin (10 μg/ml) plates per viable colony. At least three independent experiments were performed for each antibiotic concentration, and three more, with five replicas each, for the most mutagenic concentrations were performed. For the experiments with the *recA* mutants, five independent experiments were performed for each concentration.

3.9 Effects of antibiotics on *recA* expression

To qualitatively assess the antibiotic-mediated induction of transcription from the *recA* promoter the strain ME12 containing the pSC101-PrecA::GFP reporter plasmid was used. A 100 μl aliquot of an overnight culture was inoculated into LB soft agar (0.7% agar) and spread onto LB plates. Antibiotic-containing filter discs were deposited onto the agar and plates were visualized through a blue-light lamp after 24 hours of incubation at 37^{o}C. Discs with mitomycin-C (10 mg), a known inducer of the SOS system, or without antibiotic were used as a positive or negative control, respectively.

3.10 Effects of antibiotics on cell morphology

The effect of low concentrations of antibiotics on cell morphology was studied by direct observation of the treated cultures under an Olympus BX61 microscope. Aliquots (2 mL) of exponentially growing ME12 cells (10^8 cells/ml) were incubated with different antibiotics for 4 hours at 37^{o}C with shaking (250 rpm). After 4 hours of treatment,

2 μl from each culture was used to prepare samples. These samples were scanned and photographed under the microscope with an UplanF1 100 NA 1.30 oil immersion objective.

3.11 Statistical analysis

Statistical evaluation was done by using the Mann-Whitney *U-test* when two groups were compared. Differences were considered significant when *P-values* were less than 0.05.

MICs of antibiotics for ME12 and ME12recA were determined according to CLSI recommendations (Clinical and Laboratory Standards Institute, 2008), except that the bacterial inocula were identical to those used in all subsequent mutagenesis experiments. Antibiotics tested for stimulation ofmutation were used at different concentrations around their MICs.

3.12 Generation and screening of *M. smegmatis* ΦMycoMarT7 insertion library

Transposon ΦMycoMarT7, a mariner-based system, was used to obtain a *M. smegmatis* mutant library of random insertions. The isolation of a thermo-sensitive transposon phage and preparation of high-titre phage stock have been described in the literature [Sassetti et al., 2003]. For transduction, *M. smegmatis* mc^2 155 cultures were washed with MP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgSO4 and 2 mM CaCl2), mixed with the phage stock at a multiplicity of infection of 1:10 and incubated (37^oC, for 3 hours). Mycobacterial cells were then plated on Middlebrook 7H10 agar supplemented with kanamycin (25 μg/ml). The insertion mutants were isolated as kanamycin-resistant colonies after 3 days of incubation at 37^oC. Approximately 11.000 clones were picked randomly and inoculated into individual wells in 96-well microtitre plates containing Middlebrook 7H9 medium.

To identify rifampicin-resistant mutants (*M. smegmatis* wildtype, MIC 2 μg/ml),

each *M. smegmatis* transposition mutant was replicated onto Middlebrook 7H10 agar plates supplemented with several rifampicin concentrations (5, 10 or 20 μg/ml). Strains that showed resistance to the drug were retested twice to select only those able to grow on rifampicin-containing plates. To confirm the resistance phenotype for the selected mutants, the MIC of rifampicin was determined by the *broth microdilution* method. In order to define antibiotic resistance, it has been proposed that mycobacterial strains with a resistance ratio greater than 8 (defined as the MIC for the test strain divided by the MIC for the wild-type strain) are considered as resistant ones [Inderlied and Nash, 2005]. Thus, those mutants being able to grow on 20 μg/ml rifampicin (equivalent to 10-fold the MIC of rifampicin for the parental strain) were selected as candidates for further study.

3.13 Localization of ΦMycoMarT7 transposon insertion site

To determine the insertion position of the transposon cassette in the *M. smegmatis* chromosome, purified genomic DNA from each resistant mutant was digested with *BamHI* and religated with T4 ligase. Self-ligated plasmids contained the entire transposon plus flanking chromosomal DNA next to the insertion site. Re-circularized plasmids were electroporated into *E. coli* DH5$\alpha\lambda pir$116 and selected on LB with kanamycin (50 μg/ml). After purification, plasmids were sequenced using a MycoMar-specific primer that hybridizes next to the transposon/chromosome junction. The DNA sequences adjacent to each insertion site were compared with the *M. smegmatis* genome sequence to identify the interrupted gene.

3.14 Generation of *trkA* knockout mutant strains

The *M. smegmatis trkA* deletion was obtained by allelic replacement as described in the literature [Menzies et al., 2009]. Briefly, 1.0-kbp fragments up- and down-stream of the target gene were amplified by PCR with appropriate primers, 5' fragment

(DeltrkA5'F and DeltrkA5'R) and 3' fragment (DeltrkA3'F and DeltrkA3'R). Both fragments were cloned in-frame into the p2NIL vector. The resulting plasmid was digested with *PacI* to insert a *hyg sacB lacZ* cassette from pGOAL19 and verified by sequencing. The plasmid that harbored an in-frame deletion of the target gene, termed p2NIL-$\Delta trkA$, was introduced into *M. smegmatis* wild-type strain mc^2 155 and plated on Middlebrook 7H10 agar supplemented with kanamycin (25 μg/ml) and hygromycin (50 μg/ml). Once single-crossover clones were obtained, they were grown in Middlebrook 7H9 broth without antibiotics to allow a second crossover event. Finally, cultures were diluted and counter-selected on 7H10 plates containing 10% sucrose. Candidate colonies were tested for kanamycin and hygromycin sensitivity and analyzed by PCR to confirm the unmarked deletion of *trkA*.

3.15 Generation of *ich* knockout mutant strains

Similar to the generation of *trkA* knockout mutant strains ($\Delta trkA$ mutant), the fragments up-and down-stream of the *ich* gene were amplified with primers, 5' fragment (Delkch5'F and Delkch5'R) and 3' fragment (Delkch3'F and Delkch3'R). Then, they were cloned into the p2NIL vector. The resulting plasmid was inserted a *hyg sacB lacZ* cassette and verified by sequencing. Final plasmid p2NILΔich, then, was introduced into *M. smegmatis* wild-type strain mc^2 155 and colonies of a mutant with the unmarked deletion of *ich* ($\triangle ich$ mutant) were selected as described above.

3.16 Generation of *kch-trkA* double knockout mutant strains

In order to get the *ich-trkA* double knockout mutant strain, plasmid p2NIL-Δich was introduced into the *M. smegmatis* $\Delta trkA$ mutant. The double mutant $\Delta ich - \Delta trkA$ was selected in the same way as to obtain the other single mutants Δich and $\Delta trkA$.

3.17 Complementation of mutant strains

A plasmid carrying the wild-type *trkA* gene was constructed to restore expression of the gene in the $\triangle trkA$ mutants. *trkA* was amplified by PCR from *M. smegmatis* wild-type genomic DNA, using the forward primer *trkAF* and the reverse primer *trkAR*. The PCR product was digested with *NdeI* and *PstI* and cloned directly into the pVV16 vector [Telenti et al., 1993] to generate the complementation plasmid. The resulting plasmid, pVV16-*trkA*, was introduced into the *M. smegmatis* $\triangle trkA$ mutant by electroporation. Transformants were selected on Middlebrook 7H10 agar supplemented with kanamycin (25 μg/ml) and hygromycin (50 μg/ml).

The *ich* gene was amplified with oligos *ichF* and *ichR*. Then the PCR product was digested with *NdeI* and *PstI* and coloned into the pVV16 vector. The pVV16-*ich* was electroporated into the *M. smegmatis* $\triangle ich$ mutant. Transformants were selected on Middlebrook 7H10 agar supplemented with kanamycin (25 μg/ml) and hygromycin (50 μg/ml).

3.18 Growth curves and competition experiments

M. smegmatis wild-type and mutants were cultured overnight and diluted ($OD_(600)$ 0.05) in 5 ml Middlebrook 7H9 medium, and growth curves performed in triplicate for 24 hours at OD_{600}.

Fitness of *M. smegmatis* strains was quantified in vitro by competition assays in which overnight cultures of wild-type (rifampin-sensitive) and the $\triangle trkA$ mutant (rifampin-resistant) strains were diluted and adjusted to inoculate the same cell numbers in three mixed cultures of 5 ml 7H9 medium (initial $OD_(600)$ 0.05). Competition cultures with the drug-resistant and drug-sensitive strains were incubated (37^oC) and 10-fold serial dilutions plated after 0, 10 and 20 hours on Middlebrook 7H10 agar alone or with rifampin (2 μg/ml). Colony-forming units (CFU) of the rifampin-resistant $\triangle trkA$ strain were counted on drug-containing plates, while CFU counts of the wild type were obtained after subtracting CFU for the $\triangle trkA$ mutant from that for total

viable cells on drug-free plates. The *trkA* mutant fitness was measured as the ratio of the number of generations of the rifampin-resistant strain relative to that of the wild type strain.

3.19 MIC determination

MIC for the *M. smegmatis* wild-type and mutant strains were determined in triplicate in Middlebrook 7H10 agar supplemented with two-fold increasing concentrations of antibiotics. Each strain was grown in Middlebrook 7H9 broth to logarithmic phase. Cultures were diluted to yield a standard inoculum containing 10^6 CFU/ml, and approximately 10^4 viable cells were plated on drug-containing and on drug-free plates. The MIC of an antibiotic was defined as the lowest concentration at which no growth was visible after 3-5 days under incubation. To analyze the effect of antibiotics on cell survival, the number of CFU on drug-containing plates was divided by that for viable cells on drug-free plates.

3.20 Membrane potential assay

For the membrane potential assay, three cultures of *M. smegmatis* wild-type and mutant strains were grown to late logarithmic phase. Cultures were washed and adjusted to the same number of cells (10^7 CFU/ml). Cell membrane potential was estimated using the fluorescent probe rhodamine 123 (Sigma), [Morlock et al., 2000] a lipophilic cationic molecule that is taken up into mycobacterial cells in response to the level of the electrical potential [Cummings and Segal, 2004]. Inside the cells, probe fluorescence is quenched.

To measure the degree of fluorescence decay, rhodamine 123 was added to samples at a final concentration of 0.5 μg/ml. Time courses of fluorescence decay were analyzed by measuring the fluorescence decrease in the samples over a ten minutes long period using a Tecan infinite F200 spectrofluorimeter (480 nm excitation and 530 nm emission). The rate of fluorescence decay was normalized to the initial fluorescence

and the fluorescence decay of the probe itself.

Moreover, the membrane potential was also measured via monitoring fluorescence of rhodamine 123. This dye was added to the samples at a final concentration of 0.5 μg/ml. Then the fluorescence was measured in Tecan infinite F200 spectrofluorimeter (480 nm excitation, 530 nm emission). Wild-type probe with additional valinomicin was used as a control.

3.21 Estimating spontaneous mutant frequencies

For spontaneous mutant frequency estimation, approximately 10^3 cells from overnight cultures were inoculated into three tubes with 5 ml Middlebrook 7H9 medium and incubated (37^{o}C). When cultures reached late logarithmic phase, cell aliquots were plated on Middlebrook 7H10 agar supplemented with rifampin (50, 75 or 100 μg/ml) and incubated (37^{o}C, 3 to 5 days). Serial dilutions from cultures were also plated on 7H10 agar without antibiotic to estimate the number of viable cells. Mutant frequency was measured as the average number of rifampin-resistant colonies divided by the average number of viable cells.

Chapter 4

Results

4.1 Antibiotics induce mutagenesis in *E. coli*

4.1.1 Effect of different concentrations of antimicrobials on *E. coli* mutagenesis

In principle, mutagenic activities of antimicrobials are expected to occur within a window of concentrations very close to the MIC (peri-MIC), because higher concentrations will kill or stop the growth of most of the cells in the population and lower ones will have not stimulatory effect [Couce and Blazquez, 2009]. In this work, we investigated the mutagenic effect of thirteen antimicrobials at peri-MIC concentrations on the strain ME12, a MG1655 derivative, by evaluating the appearance of mutants resistant to rifampicin and fosfomycin. We used the strain ME12 for consistence, because it was used to study the effect of the same antibiotics on homologous recombination [Lopez et al., 2007, Lopez and Blazquez, 2009]. This strain shows a spontaneous frequency of rifampicin-resistant mutants of $2x10^{-7}$ and of fosfomycin-resistant mutants of $1x10^{-6}$ (not shown). Table 4.1 shows the MIC of each antimicrobial under our experimental conditions for the strain ME12. The mutagenic effect was tested for five different concentrations, including two below and two over the MIC and the MIC (i.e. 1/4xMIC, 1/2xMIC, MIC, 2xMIC and 4xMIC). The concentration of each antimicrobial producing the highest effect was re-tested using five independent replicates to con-

Antibiotic	**ME12**	**ME12△*recA***
Ampicillin (Amp)	1	1
Ceftazidime (Caz)	0.25	0.12
Imipenem (Imi)	0.12	0.12
Fosfomycin (Fos)	0.06	0.03
Ciprofloxacin (Cip)	0.12	0.007
Trimethoprim (Tri)	0.5	0.25
Sulfamethoxazol (Sul)	256	256
Tri/Sul (1/19)$^{\sharp}$	0.5/9.5	0.25/4.75
Colistin (Col)	8	2
Tetracycline (Tet)	0.5	0.5
Gentamicin (Gen)	0.5	0.5
Rifampicin (Rif)	2	2
Chloramphenicol (Chl)	2	2

Table 4.1: Minimal inhibitory concentrations (μg/ml) of the antimicrobials used in this study against the wild-type strain ME12 and its *recA*-derivative.$^{\sharp}$: The proportion trimethoprim/sulfamethoxazol is 1/19 as indicated by the EUCAST http://eucast.www137.server1.mensemedia.net/clinical_breakpoints

firm the results. Ten antimicrobials (ampicillin, ceftazidime, imipenem, fosfomycin, ciprofloxacin, trimethoprim, sulfamethoxazole, trimethoprim/sulfamethoxazole, colistin and tetracycline) produced statistically significant increases ($P \leq 0.05$) in the mutant frequency when it was calculated for rifampicin-resistance, with maximal increases of 3.4, 2.2, 3.0, 5.0, 2.0, 17.1, 6, 3, 8.7, 3.0 and 2.1-fold, respectively (Figure 4-1A, black bars).

The results from the other three drugs were not statistically significant ($P > 0.05$). When the mutagenic effect was studied calculating the mutant frequency for fosfomycin-resistance, eight antimicrobials (ampicillin, ceftazidime, imipenem, ciprofloxacin, trimethoprim, sulfamethoxazole, trimethoprim/sulfamethoxazole and tetracycline) produced statistically significant increases ($P \leq 0.05$) in the mutant frequency, with maximal increases of 3.6, 2.0, 2.2, 2.2, 7.7, 4.9, 7.9 and 3.0-fold, respectively (Figure 4-1B, black bars). The results from the other five drugs were not statistically significant ($P > 0.05$). Taken together, these results indicate that at least eight out of thirteen antimicrobials or combinations (those with positive results in both tests) produced increased mutagenesis levels at concentrations close to

their MICs. Interestingly, while most antimicrobials produced mild increases in mutagenesis, trimethoprim, sulfamethoxazole and the combination of trimethoprim plus sulfamethoxazole produced the highest increases in mutant frequency in both tests (rifampicin resistance and fosfomycin resistance). The resolution of filaments was verified by direct observation of samples from the different cultures under the microscope before plating, and only cultures with a proportion of filaments lower than 5% of total cells were plated. Thus, increased mutant frequency is not attributable to the presence of filamented cells in the treated cultures. A description of the effect of antibiotic treatment on cell morphology can be found below.

As the number of viable bacteria in the inoculum (after antibiotic treatment) might affect the observed frequency of mutants, we performed experiments with different inoculum sizes of untreated ME12 cells, ranging from 10^7 to 10^9 cells. No differences were observed in the mutant frequencies among these cultures (not shown). Thus, the final number of viable cells after treatment with different drugs was not the cause of the observed antibiotic-mediated stimulation of mutagenesis.

Finally and remarkably, treatments with rifampicin or fosfomycin did not produce an increased number of rifampicin-resistant or fosfomycin-resistant mutants, respectively, thus indicating that the concentrations of these antibiotics and/or the time of exposure used in our experiments were not able to select for rifampicin-resistant or fosfomycin-resistant variants.

4.1.2 SOS induction by the different antimicrobials

In the literature, [Perez-Capilla et al., 2005, Ysern et al., 1990, Kohanski et al., 2007, Miller et al., 2004, Gillespie et al., 2005], the induction of the SOS stress response by some antimicrobials has been well investigated. However, in order to know whether the observed stimulation of mutagenesis can be linked to an SOS induction, we studied the effects of these antimicrobials on the induction of *recA* transcription. We used the disk-plate assay described in the *Materials and Methods section*. Figure 4-2 shows that ampicillin, ceftazidimie, ciprofloxacin, trimethoprim, sulfamethoxazole and trimethoprim plus sulfamethoxazole induce transcription of the *recA*::GFP fusion,

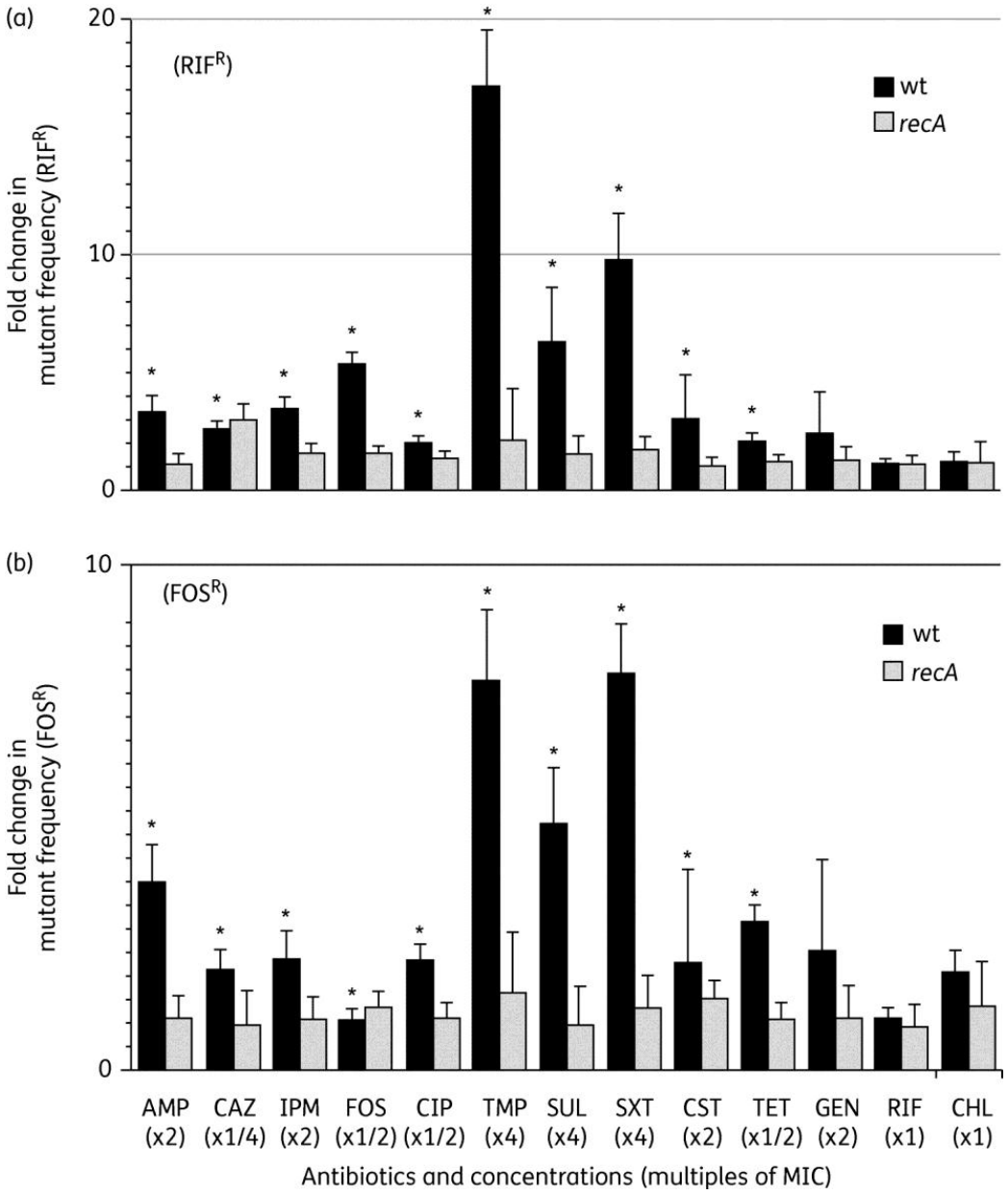

Figure 4-1: Effect of sublethal concentrations of antimicrobials on mutant frequency. Fold changes in mutant frequency of the wild-type (wt) strain ME12 (black bars) and its *recA* mutant derivative (grey bars) for rifampicin resistance (RIF^R) (a) and fosfomycin resistance (FOS^R) (b), after treatment with antibiotics. Data are relative to untreated controls (no antibiotic). Only concentrations with the highest change in mutant frequency are represented. The number in parentheses below the antibiotic indicates the concentration relative to its MIC. Values are the means of five experiments ±SD. Asterisks indicate that the fold increases relative to the untreated strain are statistically significant ($P < 0.05$), according to the MannWhitney *U-test*). AMP, ampicillin; CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin; TMP, trimethoprim; SUL, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; CST, colistin; TET, tetracycline; GEN, gentamicin; CHL, chloramphenicol.

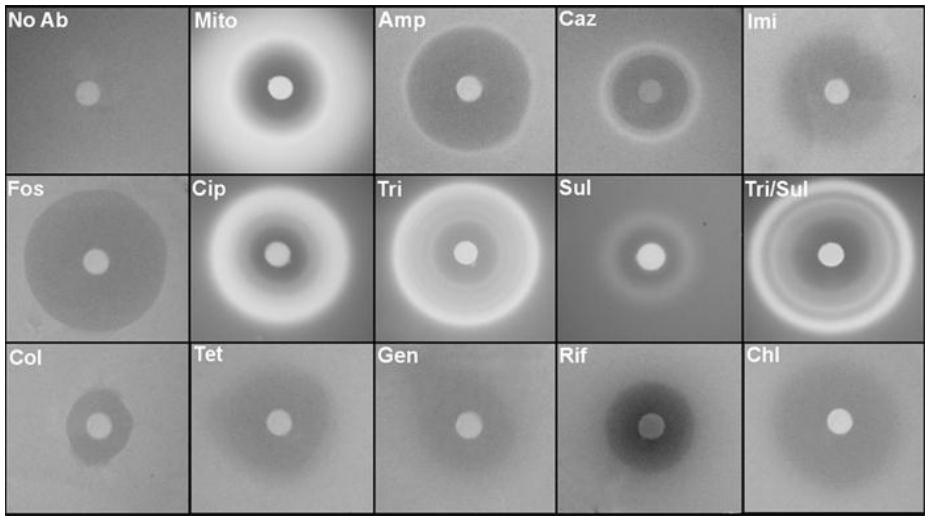

Figure 4-2: Effects of antibiotics on the transcription of the *recA*::GFP fusion after 16 hours on solid surface. 100μl of an overnight culture were inoculated into LB-soft agar (agar 0.7 %) and spread onto LB plates. Antibiotic-containing filter disks were deposited onto the gelified agar, and plates were visualized through a blue-light lamp after 16 hours of incubation at 37^{o}C. Induction is observed as a fluorescent band around the inhibition halo produced by Amp, Caz, Cip, Tri, Sul and Tri/Sul. This assay permits the exploration of the full range of antibiotic concentrations for SOS induction without knowing the most effective.The disks contain different amounts of antibiotic (g): Amp (500), Caz (10), Imi (40), Fos (100), Cip (10), Tri (300), Sul (3,200), Tri/Sul (13.5/256), Col (200), Tet (100), Gen (200), Rif (200) and Chl (300). Disks containing no antibiotic and 10 g of mitomycin-C (Mito), a known inducer of the SOS system, have been used as negative and positive controls, respectively.

with the highest induction produced by ciprofloxacin, trimethoprim and trimethoprim plus sulfamethoxazole.

4.1.3 Effects of RecA on antibiotic-mediated stimulation of mutagenesis

It has been demonstrated that ciprofloxacin and ceftazidime stimulate mutagenesis in *E. coli* through the induction of mutagenic DNA-polymerases of the SOS system [Perez-Capilla et al., 2005, Ysern et al., 1990]. Therefore, we studied the effects of the different antimicrobials on mutant frequencies in a *recA*-deficient background. As in the case of the wild-type strain, MICs of the different drugs were obtained for the *recA* strain (Table 4.1). As we expected, a strong decrease in ciprofloxacin

MIC was observed between the wild-type and its *recA*-deficient derivative. Also, a slight decrease was observed in the MICs of ceftazidime, fosfomycin, trimethoprim, trimethoprim/sulfamethoxazole and colistin.

The effects of drugs on the mutant frequency of the *recA*-deficient derivative were studied with different peri-MIC concentrations, including the MIC itself. The mutagenesis stimulated by ampicillin, imipenem, ciprofloxacin, trimethoprim, trimethoprim plus sulfamethoxazole and tetracycline is abolished in the *recA* background (Figures 4-1). Gray bars show the results with concentrations equivalent to the most mutagenic in the *recA*-proficient strain. None of the tested concentrations showed an increased mutagenesis in the *recA*-deficient strain (except ceftazidime in the rifampicin-resistance test). Therefore, RecA is absolutely necessary for the stimulation of mutagenesis by the eight antimicrobials with the positive results in both rifampicin-resistance and fosfomycin-resistance tests.

4.1.4 Effects of mutagenesis-stimulating concentrations of antimicrobials on cell morphology

We studied the effects of peri-MIC concentrations of antimicrobials on cell morphology. The most stimulating concentrations are shown in Figure 4-3. Figure 4-3A shows that ampicillin, ciprofloxacin, trimethoprim, sulfamethoxazole and trimethoprim plus sulfamethoxazole, and the SOS-inducer Mitomycin-C (positive control) produced, as expected, a clear filamentation of ME12 cells after 4 hours of treatments. A small cell enlargement can be seen along with tetracycline. In addition, imipenem produced the classical ball-shaped cells. We also studied the effects of the corresponding antimicrobial concentrations on the *recA*-deficient strain. Figure 4-3B shows that, as predicted from its mechanism of action (inhibition of the septation process via protein PBP3), ceftazidime also produced filaments in the *recA* mutant. Amazingly, ciprofloxacin and trimethoprim (and sulfamethoxazole in a lesser extent) produced filaments in the *recA* derivative, although shorter than in the wild-type. This is an unexpected result as the production of filaments by these antibiotics was believed to

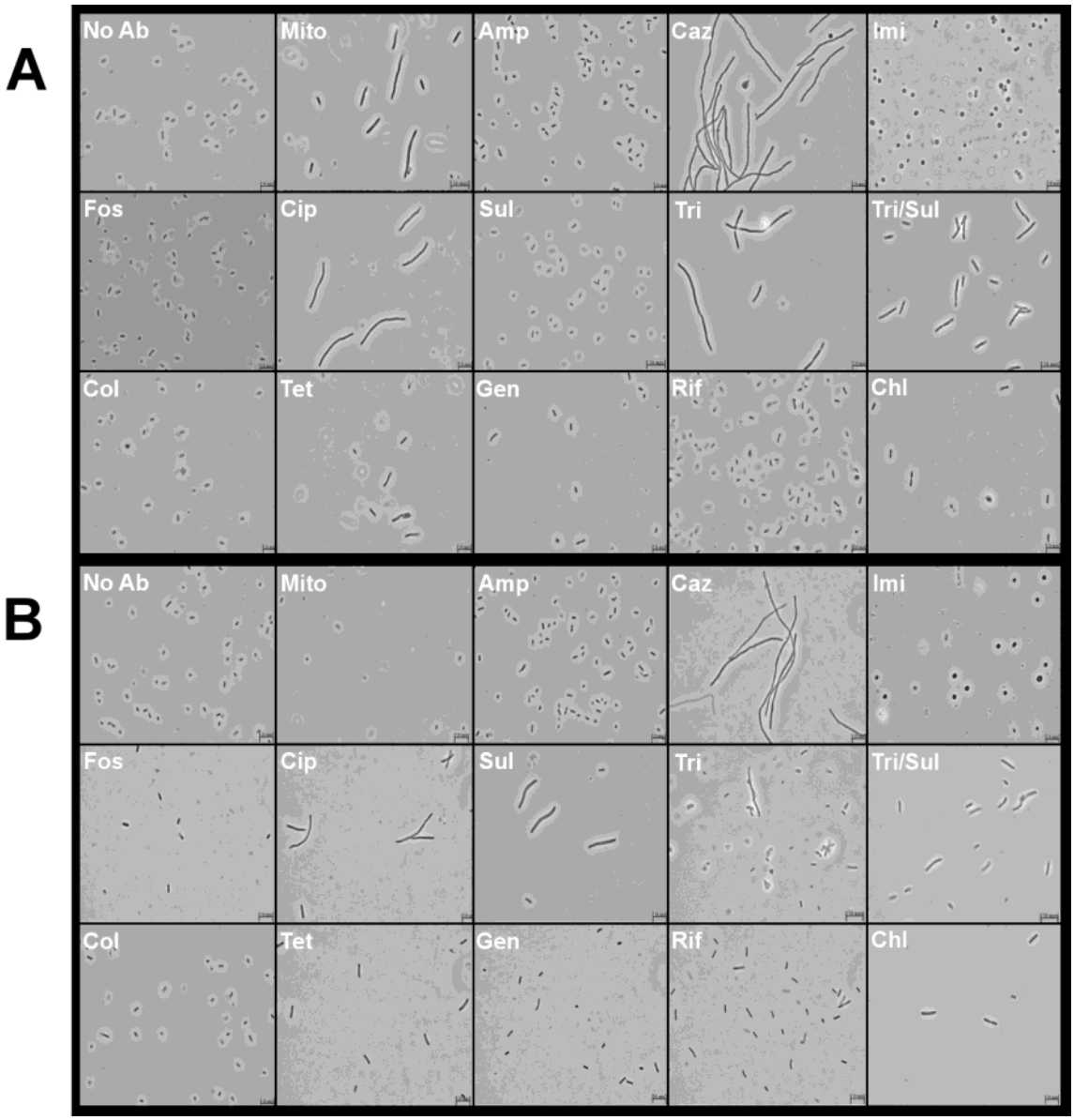

Figure 4-3: Effects of antibiotics on cell morphology. Bacterial cultures were treated for 4 hours as indicated in the Materials and Methods section. A: Effect on the wild-type strain ME12. B: Effect on the ME12 *recA* derivative. Bars at the right bottom of each image represent 10 μm.

be caused by the induction of SOS system (see below). Thus, according to our results, a *recA*-independent mechanism of filamentation can be predicted.

4.2 Antibiotic resistance in *M. smegmatis*

4.2.1 Screening for mutants

Generation transposon library

Transposon mutagenesis can be considered as a useful tool for bacterial genetic studies because of the following reasons. First of all, an antibiotic marker of transposon is an indicator that distinguishes mutants from wild-type. Secondly, transposon marks its insertion sites, so the location of the changes can be easily isolated. Thirdly, transposons can be constructed in order that recipient strains contain only a single mutation. For example, the transposons can be constructed with the transposase gene located outside of the transposon boundiries. When transposition occurs, the transposase gene is lost together with the phage vector. Therefore, once the element inserts in the chromosome it cannot transpose to a new site. Finally, these elements can be designed to discover useful properties of bacteria such as the ability to form transcriptional or translational fusions [Parish and Brown, 2011].

We have used ΦMycoMarT7 containing *Himar1*-based mariner transposon to produce a large number of *M. smegmatis* mutants isolated after several independent high efficient transduction events. So far, the analysis of the insertion sites from a large number of mutants has revealed that transposon mutagenesis produced stable mutants with no evidence of multiple insertions in each single strain. HimarI transposons were inserted at random TA dinucleotides sites within the genome. Therefore, there are a countable number of locations where the insertions can take place [Sassetti et al., 2001].

Because the transposon has a kanamycin resistant cassette, the insertion mutant can be selected on a medium supplemented with kanamycin 25 μg/ml; totally, we generated an extensive transposon insertion mutant library of *M. smegmatis* mc^2

155 containing about 11.000 independent insertion mutants. The large number of insertion mutants isolated by transposition covers more than two times the total number of non-essential genes predicted in the *M. smegmatis* genome, with a high probability ($P > 0.9$) that at least one insertion disrupts each target gene.

To verify the complexity of our insertion mutant library, we searched for and selected mutant strains which may have an antibiotic resistant phenotype. The main experimental results in this thesis were obtained when rifampicin is used to select the rifampicin resistant mutants.

Identification of rifampicin resistant candidates

Rifampicin is one of the most powerful first line anti-TB drugs. The mutations in the *rpoB* gene are the major mechanism of rifampicin resistance. However, as we have discussed in the introduction section, there are alternative mechanisms of rifampicin resistance which is not related to mutations in *rpoB*. Therefore, we probed for rifampicin resistant mutants that can help us to discover the alternative mechanism of resistance to rifampicin and understand in deep the role of these mutants in the response to the other factors as an effect of other antibiotics.

For identification of the mycobacterial genes implicated in rifampicin resistance, a transposon mutant library of *M. smegmatis* mc^2 155 was screened to obtain mutants with reduced rifampicin susceptibility. Each insertion mutant was replicated on Middlebrook 7H10 agar plates supplemented with rifampicin (5, 10 or 20 μg/ml). The growth of the *M. smegmatis* wild-type strain was completely inhibited at these antibiotic concentrations (MIC of rifampicin for *M. smegmatis* mc^2 155, 2 μg/ml); nonetheless, dozens of insertion mutants with increased rifampicin resistance were able to grow on plates containing 5 μg/ml rifampicin. Among them, 16 mutants grew on plates supplemented with 10 μg/ml rifampicin. The rifampicin resistance phenotype of these strains was confirmed by MIC. Lastly, the three transposition mutants with the highest degree of rifampicin resistance that grew well on 20 μg/ml rifampicin were selected for further analysis.

To identify the disrupted gene in each strain, DNA sequences for each mutant were

Locus	Identity	Number of strains	MIC to rifampicin (μg/ml)
MSMEG_1945	Ionic channel Ich	1	20
MSMEG_2771	K^+ regulator TrkA	2	20

Table 4.2: Rifampicin resistant candidates

compared with the *M. smegmatis* genome sequence as described in the materials and methods section. Inside these genes, a high proportion of transposon insertion are targeted to different gene locations as expected by random mutagenesis. However, a few genes contain overlapping insertions in the same location, demonstrating a sub-saturation level of gene inactivation. Rifampicin resistant candidates studied in this thesis are listed in Table 4.2.

4.2.2 Analysis of interested rifampicin resistant candidates

trkA

From rifampicin resistant candidates, there were two selected mutants had independent insertions disrupting the same target gene MSMEG_2771, which encodes a putative K^+ transport regulatory protein (Figure 4-4a). One mutant harbored a transposon insertion near the translation start codon (TA dinucleotide at position +7); the other carried another transposon insertion in the middle of the gene (TA dinucleotide at position +321). Both mutants showed the same level of rifampicin resistance (MIC 32 μg/ml), in accordance with a loss of gene function. Inactivation by transposition of a gene that encodes a K^+ transport protein thus confers increased resistance to rifampicin in *M. smegmatis*.

Characterization of the *trkA* gene in rifampin resistance. The target gene MSMEG2771, whose disruption increased *M. smegmatis* rifampin resistance, encodes a TrkA protein with a K^+ conductance regulatory domain (RCK). RCK proteins are subunits that control K^+ transporters in Bacteria, Archaea and Eukarya [Choe, 2002]. The core Trk system for K^+ uptake in prokaryotes consists of two components: the integral membrane K^+ translocating protein and the NAD^+/NADH-binding peripheral membrane protein TrkA [Dosch et al., 1991]. TrkA binds to the cytoplasmic portion of the $K+$ membrane transporter, where it acts as an essential factor in K^+ uptake

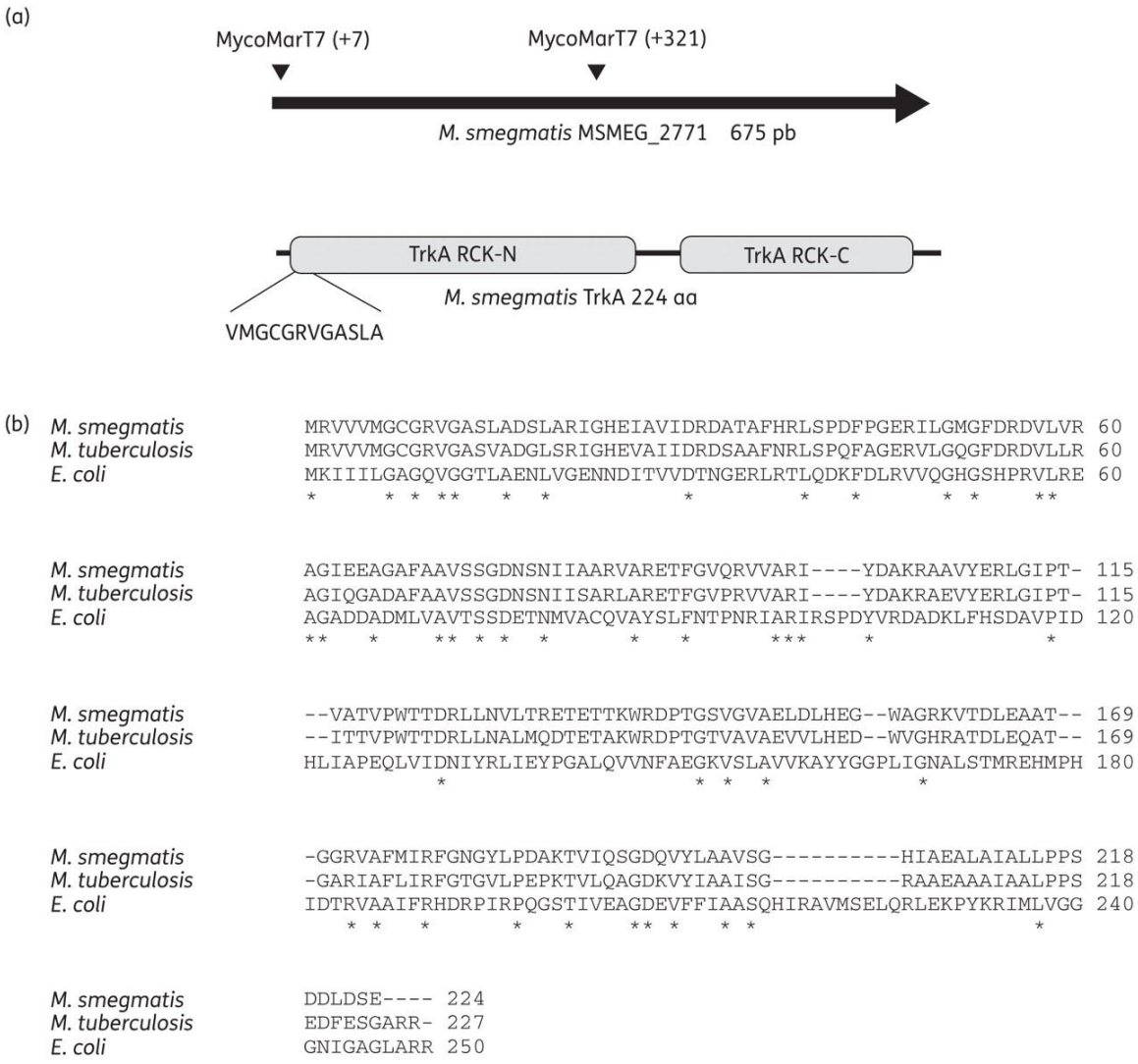

Figure 4-4: Analysis of the *M. smegmatis trkA* gene. (A) Vertical arrows indicate the location of transposon insertion in each rifampin-resistant mutant (number of base pair in the *trkA* gene)(top). Domain organization of the TrkA protein, showing the NAD(H)-binding motif sequence (bottom). (B) Alignment of TrkA sequences from *M. smegmatis* (MSMEG_2771), M. tuberculosis (Rv2691) and *E. coli.* Asterisks indicate identical residues.

[Bossemeyer et al., 1989]. In *E. coli*, TrkA-mediated K^+ transport is driven by proton motive force and also requires a high ATP concentration [Rhoads and Epstein, 1977].

Analysis of the *M. smegmatis* TrkA sequence showed that the protein consists of two tandemly arranged halves (RCK-N and RCK-C terminal; Figure 4-4A). The N-terminal subdomain was predicted to form a Rossmann fold, similar to dehydrogenase enzymes. This region has a nucleotide-binding sequence (Figure 4-4A), previously identified as a flavin adenine dinucleotide-binding motif. This characteristic motif, also present in the *E. coli* TrkA protein, binds NAD^+ or NADH with high affinity [Schlosser et al., 1993]. *M. smegmatis* TrkA protein shared considerable sequence identity with other TrkA domain proteins, such as *E. coli* TrkA N-terminal (23%) and *M. tuberculosis* CeoB (74%) (Figure 4-4B).

Deletion of the *trkA* gene decreases rifampin susceptibility in *M. smegmatis*. To establish that the loss of TrkA activity is responsible for enhancing rifampin resistance in *M. smegmatis*, we generated an in-frame $\triangle trkA$ deletion mutant. Rifampin MIC for the wild-type strain and the $\triangle trkA$ mutant were determined in 7H10 agar (Table 4.3). The *trkA* mutant showed considerable rifampin resistance (32 μg/ml), with a 16-fold higher MIC of rifampin than the wild-type strain. Being consistent with the absence of polar effects, the degree of resistance of the deletion mutant was similar to that of the two insertion mutants. Analysis of survival curves confirmed that $\triangle trkA$ was highly viable when mutant cells were exposed to antibiotic concentrations which killed the wild-type strain (Figure 4-5a).

The *trkA* gene from *M. smegmatis* wild-type strain mc^2 155 was cloned into the shuttle vector pVV16 and the resulting plasmid, pVV16-*trkA*, was introduced into the $\triangle trkA$ strain. Plasmid expression of the *trkA* gene in the deletion mutant restored rifampin susceptibility (MIC 2-4 μg/ml), but had no effect on the MIC of the wild type strain, confirming that loss of *trkA* expression was the only factor responsible for the rifampin resistance phenotype in *M. smegmatis*. These results demonstrate that *trkA* gene inactivation increases *M. smegmatis* resistance to rifampin.

Deletion of the *trkA* gene confers isoniazid sensitivity in *M. smegmatis*. Overexpression of the *M. tuberculosis* TrkA ortholog CeoB confers isoniazid resistance

Antibiotic	Molecular weight	LogS$^{\sharp}$	**MIC** (μg/ml) mc^2 155	**MIC** (μg/ml) $\triangle$*trkA*
Novobiocin	612	-4.80	16	64
Rifampin	822	-4.09	2	32
Ofloxacin	361	-2.40	0.32	0.32
Ciprofloxacin	331	-2.39	0.32	0.32
Ethionamide	166	-2.30	32	32
Ethambutol	204	-1.43	2	2
Amikacin	585	-1.07	0.8	0.4
Streptomycin	581	-0.96	0.8	0.2
Kanamycin	484	-0.72	3.2	1.6
Capreomycin	1,321	n/a	6.4	1.6
Isoniazid	137	-0.59	128	32

Table 4.3: MIC of the main groups of antimycobacterial agents for *M. smegmatis* wild-type mc^2 155 and the $\triangle$*trkA* mutant. Antibiotics are ordered by solubility values from hydrophobic to hydrophilic. $^{\sharp}$: predicted solubility according to Drugbank (http://www.drugbank.ca)

in an isoniazid-sensitive *E. coli oxy R* mutant [Chen and Bishai, 1998]. It has been suggested that TrkA binds and sequesters isoniazid, preventing antibiotic attachment to its target [Argyrou et al., 2006]. To examine the effect of *trkA* inactivation on isoniazid susceptibility, we determined the MIC of isoniazid for the wild-type strain and the $\triangle$*trkA* mutant (Table 4.3). Although *M. smegmatis* shows intrinsic isoniazid tolerance, the $\triangle$*trkA* mutant was more sensitive to isoniazid (MIC 32 μg/ml), resulting in a 4-fold decrease in MIC value compared to the wild-type strain (MIC 128 μg/ml). This result was reflected by a sharp decrease in $\triangle$*trkA* viability when the mutant cells were exposed to low antibiotic concentrations; in contrast, wild-type cells were affected only moderately, even at high isoniazid concentrations (Figure 4-5b). This result supports a function of TrkA as an element that protects mycobacterial cells from the action of isoniazid.

***M. smegmatis* multidrug susceptibility is dependent on TrkA activity.** To explore the role of TrkA in the susceptibility pattern of *M. smegmatis* to distinct antimycobacterial agents, we determined the MIC of a large number of antibiotics for the $\triangle$*trkA* mutant; Table 4.3 shows the correlation of antibiotic activity with the predicted hydrosolubility coefficients (LogS). Large hydrophobic antibiotics such as

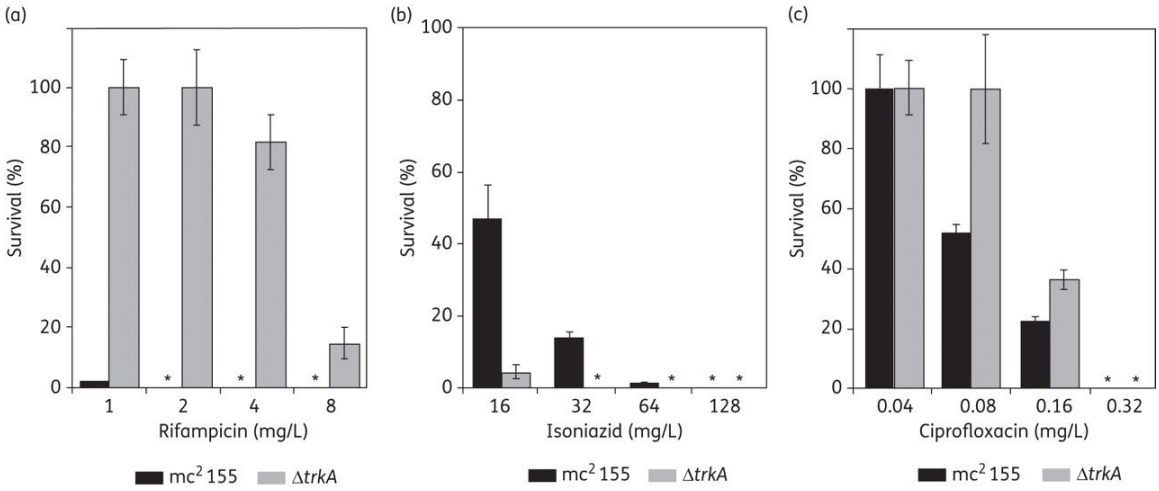

Figure 4-5: Survival (%) of *M. smegmatis* mc^2 155 wild-type and the $\triangle$*trkA* mutant under different concentrations of antibiotics. Percent of survivors of the wild-type (black bars) and the $\triangle$*trkA* mutant (grey bars) to rifampin (A), isoniazid (B), ciprofloxacin (C) and streptomycin (D) are shown. Survivors were determined by plating serial dilutions on Middlebrock 7H10 agar plates with the indicated antibiotic concentrations, and represented as the percent of surviving cells (viable cells determined on antibiotic-containing plates divided by total viable cells x100). The asterisks indicate lack of growth on plates with the indicated antibiotic concentrations, representing in all cases a survival of $\leq 0.0001\%$).

rifampin penetrate cells by passive diffusion; thus, reduced permeability to hydrophobic drugs could be responsible for the resistance phenotype in the *trkA* mutant. The MIC of novobiocin, another highly hydrophobic drug, was increased 4-fold for the $\triangle$*trkA* mutant compared to that of the wild-type strain (Table 4.3). Less hydrophobic drugs with a large apolar core, such as fluoroquinolones (ciprofloxacin, ofloxacin), were also less effective against the *trkA* mutant (Figure 4-5c), although there was no change in the MIC.

In prokaryotes, cationic antimicrobial peptides, which bind to the negative charge on the cytoplasmic membrane surface to disrupt the membrane, inhibit *trkA* mutants more efficiently than wild-type strains [Parra-Lopez et al., 1994, Chen et al., 2004]. Aminoglycosides, another group of positively charged, very hydrophilic antimycobacterial drugs, killed the $\triangle$*trkA* mutant more efficiently than the *M. smegmatis* wild-type strain. All aminoglycosides tested (kanamycin, amikacin, streptomycin and capreomycin) showed lower MIC values for the $\triangle$*trkA* mutant than those of the wild-

type strain, ranging from a 2-fold to 4-fold decrease (Table 4.3). *M. smegmatis* $\triangle$*trkA* susceptibility to small hydrophilic drugs (ethambutol, ethionamide) or β-lactams was unchanged.

These data indicate that *trkA* deletion affected the drug susceptibility pattern of *M. smegmatis*, increasing resistance to hydrophobic antibiotics and sensitivity to hydrophilic cationic agents. This suggests TrkA may influence the *M. smegmatis* permeability barrier to antibiotics as a function of the chemical nature of the drug.

Growth, K^+ requirement and fitness in the *M. smegmatis trkA* mutant. Growth curves of *M. smegmatis* wild-type strain mc^2 155 and the $\triangle$*trkA* mutant show a notable growth defect of the deletion strain (Figure 4-7a). The mutant had a lower growth rate during the logarithmic phase than the wild-type strain, although both showed the same cell density at stationary phase. The growth of the $\triangle$*trkA* strain was restored when additional K^+ was supplied to the medium (Figure 4-7b). Addition of KCl (10 to 100 mM) stimulated the growth of the $\triangle$*trkA* mutants but had no effect on wild-type cells; at 200 mM KCl, the growth of the mutants and wild-type strains was the same. These results suggest a reduced K^+ uptake due to lack of TrkA-mediated transport in the $\triangle$*trkA* mutant, and that it requires an additional K^+ supply to counterbalance its growth defect.

Resistance to antibiotics often has a fitness cost when the antibiotic is absent, shown by the reduced growth of mutants relative to the wild-type strain in mixed cultures [Andersson and Levin, 1999]. Competition experiments have shown that all rifampin resistance mutations in the *rpoB* gene impose a cost in mycobacteria [Billington et al., 1999, Gagneux et al., 2006], we thus examined whether *trkA* inactivation had deleterious effects on bacterial fitness. As stated above, *trkA* inactivation produces a notable growth defect. As expected, competition assays showed that the wild-type was strongly outcompeted by the rifampin-resistant mutant during exponential growth in mixed cultures. In these experiments, the relative fitness value of the $\triangle$*trkA* mutant was 0.77 (Table 4.4).

TrkA is necessary for pH homeostasis in *M. smegmatis*. K^+ uptake maintains intracellular ionic balance in prokaryotes [Epstein, 2003]. K^+ accumulation

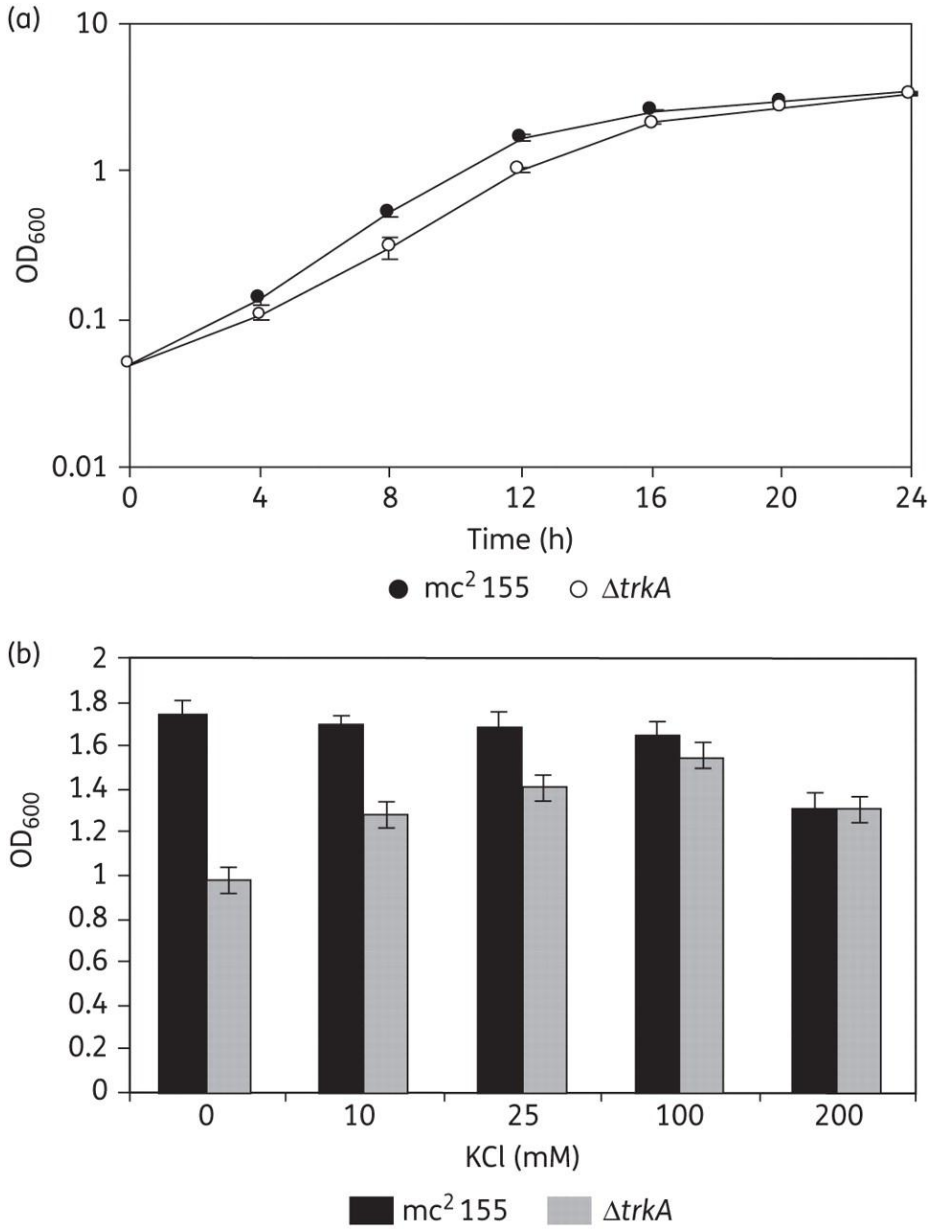

Figure 4-6: Growth curves and K^+ effect on growth of mc^2 155 and its $\triangle$*trkA* derivative. (A) Growth curves of *M. smegmatis* wild-type mc^2 155 (closed circles) and its $\triangle$*trkA* deletion mutant (open circles). (B) Effect of K^+ addition (10-200 mM KCl) on growth of mc^2 155 (black bars) and $\triangle$*trkA* (grey bars). The ability of high K^+ concentrations to restore growth of the $\triangle$*trkA* mutant was measured in cultures at mid-exponential phase (12 h). Values, optical density at 600 nm, (OD_{600}) are mean$\pm$SD of three experiments.

	Competition cultures		
Strain	No. of divisions	Doubling time (hours)	Relative fitness
mc^2 155	6.87	2.91	1.00±0.02
△*trkA*	5.32	3.76	0.77±0.02

Table 4.4: Fitness of *M. smegmatis* mc^2 155 and its △*trkA*-derivative during the logarithmic growth phase. Strains were grown in mixed cultures until late logarithmic phase (three independent experiments). The number of divisions and the doubling time of each strain were obtained from CFU counts on drug-containing and drug-free plates. The △*trkA* mutant fitness was measured as the ratio of the number of generations of the rifampin-resistant strain relative to those of the wild type strain.

is essential for intracellular pH homeostasis, and loss of K^+ uptake can thus increase sensitivity to acidic conditions. We studied *trkA* knockout mutant growth over a range of pH values (pH 5-8; Figure 4-7A). At neutral pH, the △*trkA* mutant's growth was reduced compared to that of the wild-type strain. At acid pH, the mutant's growth was severely impaired, while the wild-type strain growth was reduced only slightly. Wild-type cells were able to grow even at pH 5, at which the △*trkA* mutant growth was completely inhibited. In contrast, at alkaline with pH equal to 8, △*trkA* mutant growth was nearly identical to that of wild-type strain, with no visible growth defect. The reduced growth of the △*trkA* mutant at acid pH was compensated when supplementary K^+ was added to the medium. For instance, Figure 4-7B shows how different concentrations of K^+ counterbalance the growth defect of the △*trkA* mutant at pH 5.5. These results suggest that TrkA-dependent K^+ uptake counteracts the decrease in intracellular pH when cells are exposed to low pH values; as K^+ tends to increase intracellular pH, the K^+ uptake requirement decreases at higher pH.

TrkA inactivation leads to hyperpolarization of the cytoplasmic membrane. PMF is an electrochemical ion gradient across the membrane, with an electrical component (△ψ inside negative) and a chemical gradient (△pH, inside alkaline). The membrane is separated from the bulk aqueous phase by a barrier of electrostatic nature that could serve as a storage for protons [Mulkidjanian et al., 2006]. TrkA-dependent K^+ uptake is needed to maintain constant PMF values in prokaryotes [Bakker and Mangerich, 1981]. The K^+ uptake rate has a direct influence on the electrical membrane potential, the main contributor to PMF when cells grow in

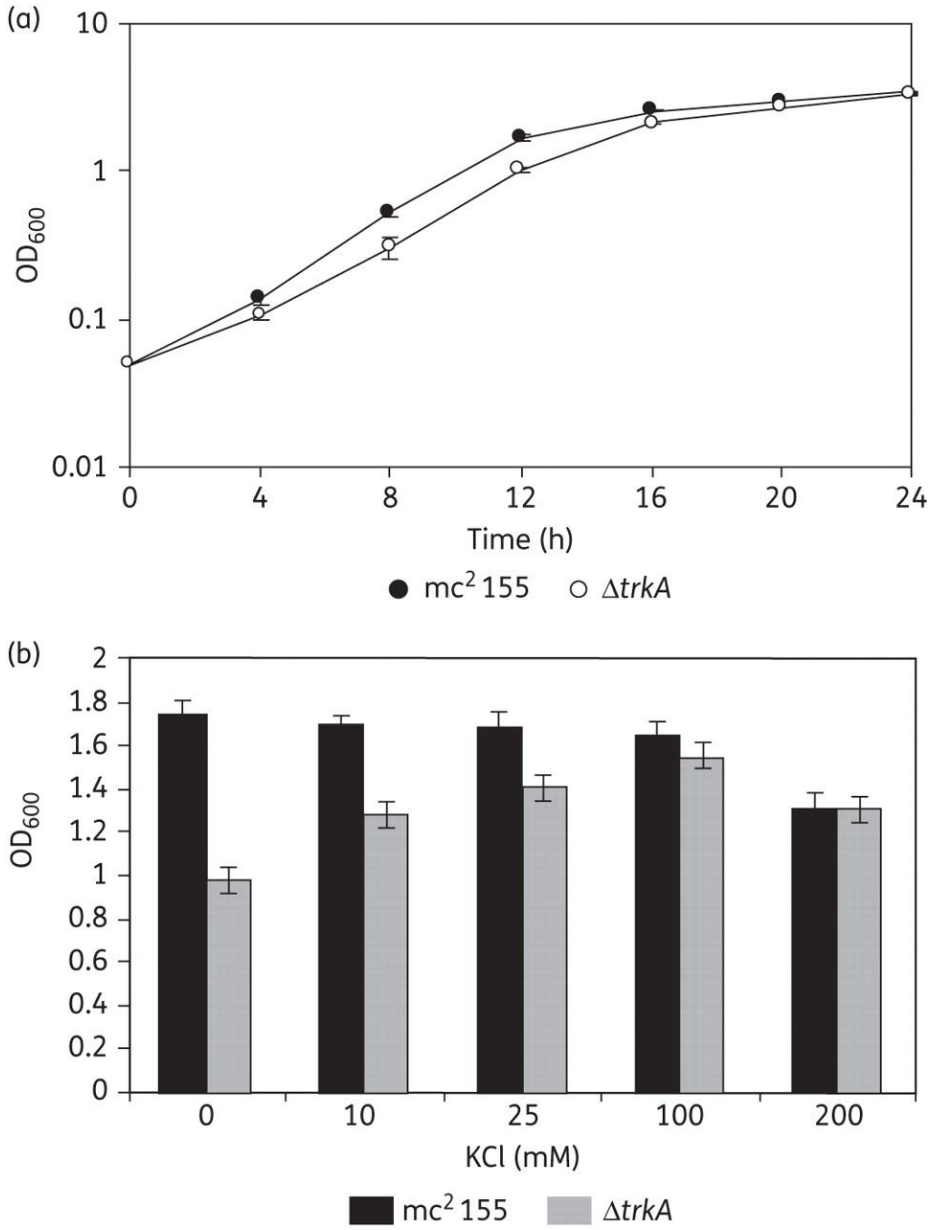

Figure 4-7: Growth curves and K^+ effect on growth of mc^2 155 and its $\triangle$*trkA* derivative. (A) Growth curves of *M. smegmatis* wild-type mc^2 155 (closed circles) and its $\triangle$*trkA* deletion mutant (open circles). (B) Effect of K^+ addition (10-200 mM KCl) on growth of mc^2 155 (black bars) and $\triangle$*trkA* (grey bars). The ability of high K^+ concentrations to restore growth of the $\triangle$*trkA* mutant was measured in cultures at mid-exponential phase (12 h). Values, optical density at 600 nm, (OD_{600}) are mean$\pm$SD of three experiments.

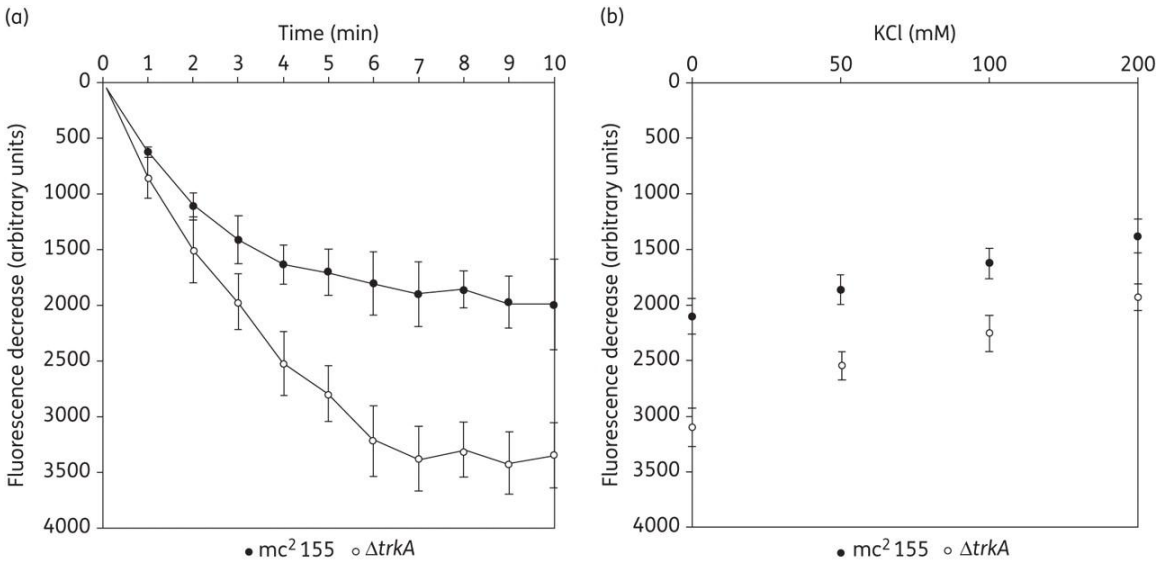

Figure 4-8: Membrane potential assay. (a) Exponentially growing cultures of *M. smegmatis* wild-type mc^2 155 (closed circles) and its $\triangle$*trkA* derivative (open circles) were incubated with rhodamine 123. The curves, monitored for 10 min, show the decrease in fluorescence emission produced by intracellular quenching of the probe. . (b) Effect of K^+ addition (50-200 mM KCl) on membrane potential of mc^2 155 and $\triangle$*trkA*. Cells were pre-incubated for 30 minutes with increasing KCl concentrations (50-200 mM KCl). Fluorescence decay was measured after 10 minutes of incubation with rhodamine 123. Values show the mean ±SD.

a neutral environment. To explore the effect of *trkA* inactivation on cell membrane electric properties in *M. smegmatis*, we estimated the electrochemical potential generated across the membrane by monitoring fluorescence quenching of rhodamine 123. Cells with higher membrane potential (increased interior negative charge) accumulate the cationic probe rhodamine 123 more efficiently, leading to the decreased intensity of fluorescence emission (28); the rate of fluorescence decay is thus proportional to the electrical membrane potential.

Compared with wild-type cells, the $\triangle$*trkA* cells showed increased rhodamine 123 uptake and, thus, enhanced fluorescence decay (Figure 4-8); these data indicate that the *trkA* mutant has a hyperpolarized cell membrane. Thus, loss of TrkA-dependent K^+ uptake is responsible for the increased electrical membrane potential in the *M. smegmatis* $\triangle$*trkA* strain. The high negative charge of the intracellular membrane in the *trkA* mutant is consistent with its previously known multidrug susceptibility

pattern (susceptibility to cationic agents and resistance to hydrophobic drugs).

Additionally, the cells were pre-incubated for 30 minutes with increasing K^+ concentrations (50, 100 and 200 mM KCl). The addition of K^+ to the medium depolarizes the bacterial membrane, leading to a strong decrease in the diffusion of the probe inside the cells and, consequently, to a reduced fluorescence decay in the samples (Figure 4-8). As expected, the *trkA* mutant maintains higher levels of membrane potential when compared with wild-type cells at each KCl concentration.

We have studied the effects of an H^+ ionophore, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), against *M. smegmatis* wild-type and the *trkA* mutant. CCCP destroys membrane potential by eliminating the proton H^+ gradient. Although the *M. smegmatis trkA* mutant had a hyperpolarized membrane, the susceptibility of both strains to CCCP was similar (MIC 4 μg/ml). Finally, we analysed the effect of K^+ ionophore and nigericin. This compound has a very high affinity for monovalent cations, such as K^+, and catalyses the electroneutral exchange of intracellular K^+ for H^+ (antiporter). Therefore, nigericin reduces intracellular K^+ levels, disrupts membrane potential and decreases intracellular pH. As expected, the *trkA* mutant showed a hypersusceptibility to the K^+ ionophore nigericin (MIC 4 μg/ml), compared with the wild-type cells (MIC 16 μg/ml). The *trkA* mutant was unable to counterbalance the K^+ efflux due to its impaired K^+ uptake, leading to severe defects in the membrane potential and intracellular pH.

Ich

The other rifampicin candidate from our library was a mutant with an insertion in the *ich* gene which encodes a putative K^+ transport protein (Figure 4-9). The gene was inactivated by the insertion of TA dinucleotide at position +1066. Because of the loss of function of the gene, the mutant showed a rifampicin resistant phenotype with MIC around 32μg/ml.

Sequence analysis of Ich protein encoded by MSMEG_1945 reveals identity with potassium transporter protein. The gene MSMEG_1945 encodes an ionic channel protein with TrkA-N domain (K^+ conductance regulator

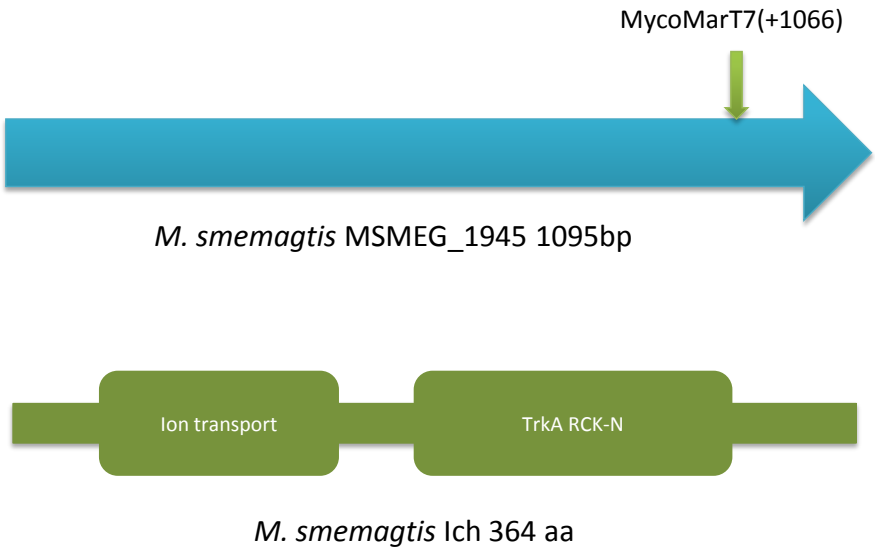

Figure 4-9: Location of transposon insertion. Vertical arrows indicate the location of transposon insertion in a rifampin-resistant mutant (number of base pair in the *ich* gene)(top). Domain organization of the Ich protein, showing the TrkA-N domain (bottom).

domain RCK-N which controls K^+ transporters in Bacteria, Archaea and Eukarya [Choe, 2002]. The analysis of the sequence of the *M. smegmatis* protein Ich encoded by MSMEG_1945 showed that the protein contains two domains: one is ion transporter locating in the membrane; the other is TrkA-N domain locating on cytoplasm. Moreover, search for the homologues of this protein by protein blast (BLASTp) revealed identity with proteins implicated in the potassium transport. We found that the Ich protein has considerable sequence identity with the protein tranmembrane cation transporter in *M. tuberculosis* (70%) with Kch of *M. massiliense* (74%) and Kch of *E. coli* (22%). In *E. coli* Kch protein is a potassium transporter similar to a group of eukaryotic K^+ channel proteins [Milkman, 1994]. It takes part in the regulation of membrane potential under certain stress and its overproduction leads to the increase of potassium permeability of cells [Kuo et al., 2005, Ungar et al., 2001, Kuo et al., 2005].

Deletion of *ich* increases resistance of M.smegmatis to rifampicin. Our analysis of insertion mutants of *ich* from our insertion library showed that the mutants with interruption in *ich* increased their resistance to rifampicin with MICs over 20

mg /ml. To avoid polar effects of the result, an in-frame knock out *ich* mutant was constructed. The MICs to rifampicin of the wild type strain and the *ich* deletion strain were determined in Middlebrook with MIC roughly 32 μg/ml. Meanwhile, rifampicin MIC of wild type strain was only 2 μg/ml that was 16 folds lower than rifampicin MIC of *ich* mutants.

Plasmid pvv16 with the cloned *ich* gene was transformed into Ich knock out mutant. The expression of *pvv16-ich* in the mutant strain recovered the phenotype of wild type. This result demonstrates that deletion of *ich* gene may be one of the key factors of resistance to rifampicin in *M. smegmatis*.

Mutant with *ich* deletion increase the susceptibility to isoniazide and ethionamde. Recent results by [Argyrou et al., 2006] suggested that TrkA binds and isolates isoniazid. Our previous work further showed that mutants lacking of the *trkA* gene became isoniazid susceptibility in *M. smegmatis* with MIC 64 μg/ml [Castaneda-G'arcia et al., 2011]. As *ich* contains TrkA domain, it may also have tight relations with isoniazid susceptibility like *trkA* does. Therefore, in this work, we performed several analyses to reveal the effect of *ich* together with *trkA* on isoniazid.

First, besides *ich* deletion mutant, we created a double mutant with deletion of both genes *ich* and *trkA*. Subsequently, MICs to isoniazid of *ich* deletion mutant and double mutant *ich-trkA* were acquired. While wild type strain of *M. smegmatis* showed resistances to isoniazid with MIC 128 μg/ml, both *ich* deletion and *ich-trkA* mutants were considerably more susceptible to isoniazid with MICs 8 μg/ml and 2 μg/ml respectively. Comparing to the acquired MIC at 64 μg/ml on the mutants lacking of the *trkA* gene alone, these numbers are significantly smaller.

Ethionamide is structurally similar to INH and also is an inhibitor of mycolic acid biosynthesis. The MICs to ethionamide of Δich and Δich-$\Delta trkA$ were determined. We found that both of mutants were more susceptible to ethionamide than wild-type. Indeed the obtained MICs were 16 μg/ml and 8 μg/ml for Δich and Δich-$\Delta trkA$, respectively; when the MIC of wild-type was 64 μg/ml. This result suggested that these genes have an important role in the defense mechanism of *M. smegmatis* from isoniazid and ethionamide.

	MICs (μg/ml)			
Antibiotics	mc^2 155	$\triangle ich$	$\triangle ich$-$\triangle trkA$	$\triangle ich$-*pvv16 ich*
Novobiocin	32	64	64	32
Rifampicin	4	32	32	8
Ofloxacin	0.32	0.32	0.32	0.32
ciprofloxacin	0.32	0.32	0.32	0.32
Ethionamid	32	8	4	32
ethambutol	2	2	2	2
Amikacin	0.8	0.4	0.4	0.8
streptomycin	0.8	0,2	0,2	0.4
Kanamycin	3.2	1.6	1.6	—
Isoniazid	128	8	2	128

Table 4.5: MIC of the main group of antimycobacterial agents for *M. smegmatis* wild-type mc^2 155, the $\triangle ich$, the $\triangle ich$-$\triangle trkA$ mutants and the complementation strain of the $\triangle ich$. Antibiotics are ordered by solubility values from hydrophobic to hydrophilic.

The role of the *ich* gene in antibiotic resistance profile of *M. smegmatis*. In order to investigate the functions of *ich* and *ich* together with the *trkA* genes, MICs of mutants Δich and Δich-$\Delta trkA$ with respect to various antibiotics were determined. Table 4.5 shows the obtained results in our experiments. Hydrophobic antibiotics such as novobiocin and rifampicin are able to pass through the outer membrane to the cell by passive diffusion. Meanwhile, the penetration of hydrophilic antibiotics is significant low. Our first observation showed that the mutants were more resistant to hydrophobic antibiotics than hydrophilic antibiotics.

In particular, inactivation of *ich* and double mutants were resistant to hydrophobic antibiotics such as novobiocin and rifampicin with two-fold and eight-fold increase in MIC. Less hydrophobic antibiotics, such as fluoroquilones almost do not have any effect on mutants with no change in MICs. On the other hand, aminoglycosides with high hydrophilic properties killed the mutants more effectively than wild type strain with two-fold decrease in MIC. However, with the other small hydrophilic antibiotics, such as ethambutol and β-lactams we obtained the same MICs as wild type.

In addition, we also analyzed the viability of wild type, Δich and Δich-$\Delta trkA$ mutants on different concentrations of several antibiotics ranging from hydrophobic to hydrophilic including rifampicin, ciprofloxacin and isoniazid. Viable count of mutants

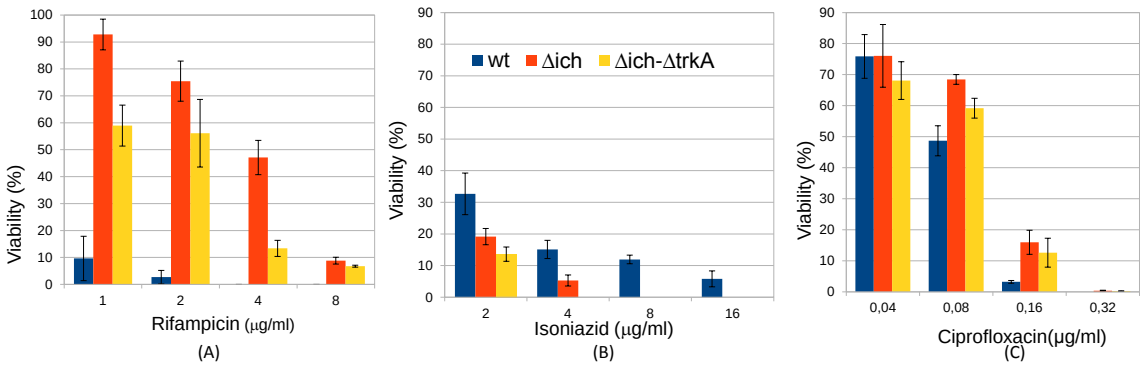

Figure 4-10: Viability (%) of *M. smegmatis* mc^2 155 wild-type, $\triangle ich$ and $\triangle ich$-$\triangle trkA$ mutants under different concentrations of antibiotics. Percentage of viabilities of the wild-type (blue bars), $\triangle ich$ (red bars) and the $\triangle ich$-$\triangle trkA$ mutant (yellow bars) to rifampin (A), isoniazid (B), ciprofloxacin (C)) at the indicated antibiotic concentrations are shown.

on different concentrations of rifampicin is much larger than viable count of wild type. This result again explains the MIC numbers of these strains shown in Table. It demonstrates that *ich* plays an important role in protection of *M. smegmatis* against rifampicin.

As we observe in Figure 4-10, although there is no change in MICs of all the strains to fluoroquilones, viable count of mutants to high concentrations of ciprofloxacin closed to the MIC is significantly larger than wild type. The results in this figure show that those antibiotics are less effective on Δich and Δich-$\Delta trkA$ mutants.

The difference between isoniazid MIC of Δich and Δich-$\Delta trkA$ mutants and wild type is supported by the viability count of those strains. In fact, in Figure 4-10 the viability of these mutants reduces dramatically when the concentration of antibiotics is increased. Meanwhile, the effect of isoniazid acting on the viability of wild type is negligible even at high concentration.

Complementation strain with expression of plasmid pvv16-IC restores all the wild type phenotypes. In summary, the reaction of Δich mutant and double mutant Δich-$\Delta trkA$ under the impact of different antibiotics demonstrates that *ich* gene together with the *trkA* gene play an important role in uptake of both hydrophobic and hydrophilic antibiotics.

Growth effect and K^+ requirement. To study the growth effect of Δich

Strain	Growth rate	doubling time (hours)
wt	0.532±0.039	1.31
$\triangle ich$	0.455±0.019	1.52
$\triangle ich$-$\triangle trkA$	0.435±0.039	1.6

Table 4.6: The growth rates and doubling time of the mc^2 155, the $\triangle ich$ and the $\triangle ich$-$\triangle trkA$ mutants. The growth rate was estimated as the maximum slope of the natural logarithm of optical densities versus time

and Δich-$\Delta trkA$ mutants, growth curves were plotted for 24 hours. The obtained growth curves of mutants and wild type are different. In particular, the Δich and Δich-$\Delta trkA$ mutants have notable lower growth rate than wild type.

According to [Andersson and Levin, 1999, Levin et al., 2000], antibiotic resistance is usually related to physiological cost for the bacteria. When bacteria are exposed to antibiotics, mutations conferring antibiotic resistance give the bacteria advantages over susceptible ones. Quantification of fitness cost is one of the methods to investigate the evolution of antibiotic resistance and its stability in a population [Pope et al., 2010]. To evaluate the fitness cost associated with antibiotic resistance the growth rate and doubling time of bacteria were calculated. From the growth curves, the growth rate was estimated as the maximum slope of the natural logarithm of optical densities at 600 nm versus time. The obtained results are showed in Table 4.6. According to that table, the growth rate of mutants is lower than the growth rate of wild-type and the mutants need more time to reach a double optical density value than the wild-type.

When K^+ was added into the medium at different concentrations the growth rate of mutants increased. However, although adding K^+ helps mutants to grow faster, it almost does not have any effect on the growth rate of wild type. This result shows that bacteria lacking of the *ich* gene require more K^+ to grow.

Loss of the Ich gene causes increase sensitivity to acidic pH. Potassium and acid-base balance were known to be interrelated. At low internal pH, cells exchange intracellular protons for extracellular K^+ [Kashket and Barker, 1977], an optimal accumulation of K^+ is essential in bacteria for the maintenance of internal pH. Therefore, loss of the K^+ retention by the disruption of the K^+ transport can

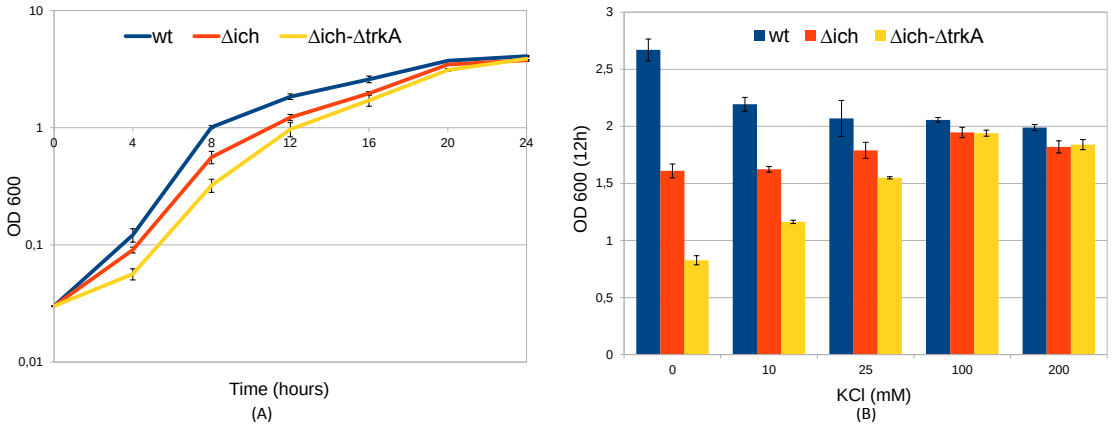

Figure 4-11: Growth curves and K^+ effect on the growth of the mc^2 155, the $\triangle ich$ and the $\triangle ich$-$\triangle trkA$ mutants. (A) Growth curves of *M. smegmatis* wild-type mc^2 155 (blue colors), $\triangle ich$ (red colors) and $\triangle ich$-$\triangle trkA$(yellow colors). (B) The effect of K^+ addition (10-200 mM KCl) on the growth of mc^2 155 (blue bars), $\triangle ich$ (red bars) and $\triangle ich$-$\triangle trkA$ (yellow bars). The ability of high K^+ concentrations to restore growth of the mutants was measured in cultures at mid-exponential phase (12 hours). Values, optical density at 600 nm, (OD 600) mean ±SD.

increase the sensitivity of bacteria when they are exposed to acidic conditions. To examine the functions of *ich* in regulation of internal pH, the $\triangle ich$ and $\triangle ich$-$\triangle trkA$ mutants were grown in liquid media with pH ranging from 5 to 8. The growth of different strains are plotted in Figure 4-11. At neutral pH, the mutants grew slower than wild type. When bacteria are grown in acid pH, the growth of mutants reduces significantly while the growth of the wild type only changes slightly. On the other hand, at alkaline pH the growth of the mutants almost reached the growth of the wild type.

In addition, the growth of mutants in the medium at pH 5.5 with different concentration of K^+ was also compared to that of wild-type (Figure 4-12). In the presence of K^+ the mutants grew better. However, even when 25mM of K^+ was added to the medium the mutants could not get the growth level of the wild-type. Only at 100mM of K^+ the growth of $\triangle ich$ mutant almost reached the the growth level of the wild-type. The aforementioned result indicates that the *ich* gene is important for potassium-dependent growth of *M. smegmatis* at low pH values.

Deletion *ich* and membrane potential. Proton motive force (PMF) is con-

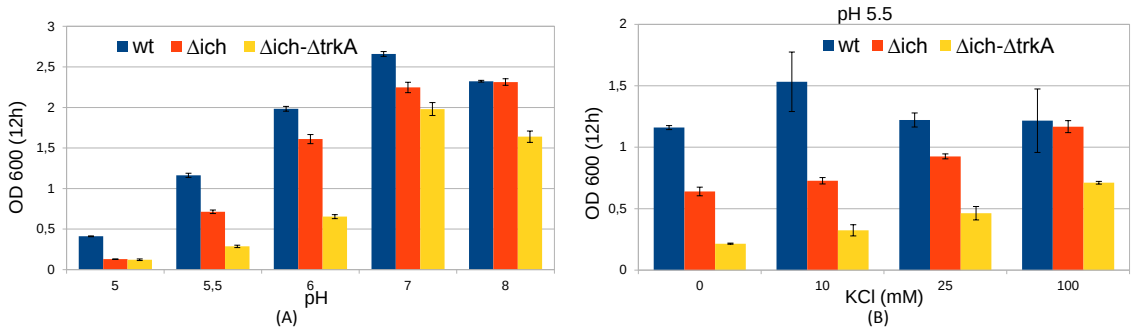

Figure 4-12: Effect of extracellular pH on growth of the $\triangle ich$ and the $\triangle ich$-$\triangle trkA$ mutants. (A) Effect of pH (5.0 to 8.0) on the growth of mc^2 155 (blue bars), $\triangle ich$ (red bars) and $\triangle ich$-$\triangle trkA$ (yellow bars) in Middlebrook 7H9 liquid medium (initial OD600 0.05). (B) Effect of K^+ addition (10-100 mM KCl) on the growth of mc^2 155 (blue bars), $\triangle ich$ (red bars) and $\triangle ich$-$\triangle trkA$ (yellow bars) at pH 5.5. Optical density (OD600) was measured after 12 hours incubation at 37°C. Values show the mean±SD

tributed by two factors: an electrical component ($\triangle\psi$, inside negative) and a chemical gradient ($\triangle$pH, inside alkaline). The uptake of K^+ directly relates to electrical membrane potential, maintaining the optimal transport of K^+ is one of the obligators for electrical membrane potential value, the main contributor to PMF when cells grow in a neutral environment [Bakker and Mangerich, 1981]. We have already studied the function of *trkA* in regulation of electrical membrane potential [Castaneda-G'arcia et al., 2011]. Following that result, we will also examine the effect of *ich* deletion on membrane potential properties in this work.

Particularly, we measured electrochemical membrane potentials across membrane by monitoring fluorescence of rhodamine 123. Cells with higher membrane potential, i.e. with charging more negatively inside, accumulated a higher number of the cationic rhodamine 123. The florescence of rhodamine 123 in the medium was monitored. The medium containing cells with high membrane potential had low fluorescence. Besides, the K^+ ionophore (valinomycin) was used for the positive control probe.

When valinomycin was added to the medium (without K^+ or with concentration of K^+ less than in the cells), it forced the cells to take K^+ out. In that way, the negative charge inside the cells increased leading to an increase of membrane potential. Therefore, cells in the positive control probe took in high number of the cationic

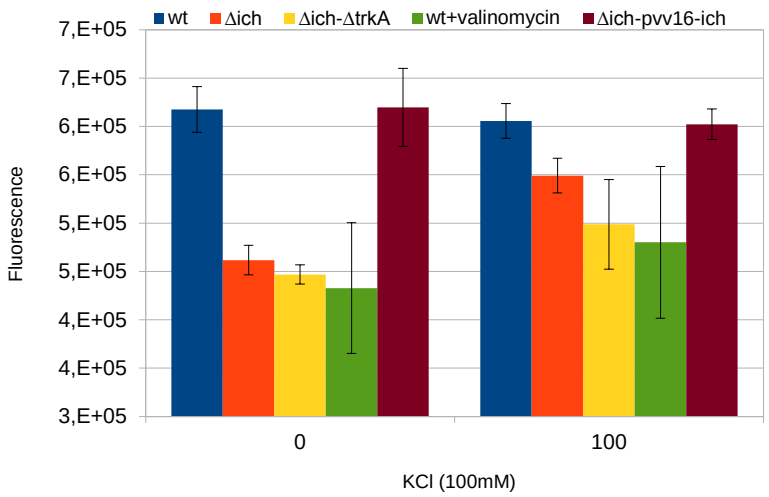

Figure 4-13: Membrane potential assay. Exponentially growing cultures (without KCl) of *M. smegmatis* mc^2 155 (blue bars), $\triangle ich$(red bars) and $\triangle ich$-$\triangle trkA$ (yellow bars) were incubated with rhodamine 123. The curves show the decrease in fluorescence emission produced by intracellular quenching of the probe. Effect of K^+ addition (100 mM KCl) on the membrane potential of mc^2 155, $\triangle ich$ and $\triangle ich$-$\triangle trkA$. Cells were pre-incubated for 30 min with increasing KCl concentrations. Fluorescence measured with rhodamine 123. Values show the mean±SD of experiments

rhodamine 123. Accordingly, rhodamine 123 in the medium decreased. At the end, compared to wild type, the medium of the positive control probe presented with lower fluorescence.

Similar to the positive control probe, mutant strains Δich and Δich-$\Delta trkA$ also showed a decrease of fluorescence. Indeed, they accumulated more rhodamine 123 and possessed higher membrane potential. The higher negative inside of the Δich and Δich-$\Delta trkA$ mutants can be considered as a reasonable explanation for the observed multidrug susceptibility, i.e. sensitivity to cationic agents and resistance to hydrophobic antibiotics.

Chapter 5

Discussion

5.1 Antibiotics and mutagenesis

Over the past six decades, the extensive usage of antimicrobial drugs has established a major impact on human-associated bacteria including both commensal and pathogens, leading to the selection and the wide spread of resistant variants. The frequent exposure of bacteria to different antibacterial agents results in the selection of preexisting resistant variants that ultimately become fixed in the population [Lederberg and Lederberg, 1952, Newcombe, 1949]. Under antibiotic stresses, many bacteria with an increased mutation rate (hypermutators) survive and become resistant to the antibiotics [Mao et al., 1997].

The induction of the SOS response by antimicrobials, such as fluoroquinolones, and its consequence, i.e. the increased mutagenesis, were described in the literature long time ago [Ysern et al., 1990]. Since the SOS response is efficiently activated by DNA-damaging agents leading to the transcriptional induction of the error-prone DNA-polymerases, it is not surprising that clear evidence of antimicrobial-induced mutagenesis was first described with fluoroquinolones [Ysern et al., 1990, Power and Phillips, 1993]. However, the induction of the SOS response, and its direct consequence, i.e. the increased mutagenesis caused by β-lactams have just been recently revealed in the literature [Perez-Capilla et al., 2005, Miller et al., 2004].

Recent work by [Kohanski et al., 2007] has demonstrated that some antimicro-

bials, defined as bactericidal, such as ampicillin, norfloxacin and kanamycin, stimulate the production of highly deleterious ROS radicals in Gram-negative and Gram-positive bacteria, which ultimately can contribute to cell deaths. On the contrary, bacteriostatic drugs do not produce such effects. Besides, it also shows that the same bactericidal drugs induce the SOS stress response and that the inactivation of RecA, a co-regulator of the response, produces an increased susceptibility to some of them, including norfloxacin, ampicillin and kanamycin [Kohanski et al., 2007, Lewin et al., 1989]. In this work, our results discovered an additional interesting fact that mutagenesis is not only induced by bactericidal drugs but also by bacteriostatic ones, and that inactivation of RecA activity abolishes the induction of mutagenesis in all cases.

Another interesting result from our investigation is that trimethoprim, either alone or in combination with sulfamethoxazole, promotes the highest increase in mutant frequency (Figures 4-1A and 4-1B). Trimethoprim prevents incorporation of thymine into bacterial DNA by inhibition of dihydrofolate reductase [Amyes and Smith, 1974]. It not only provokes the induction of the SOS response [Lewin and Amyes, 1991] but also the nucleotide pool imbalance which may affect the replication fidelity [Lewin and Amyes, 1991]. Therefore, both SOS response and nucleotide imbalance might act synergistically to the increase of mutant frequency. The other interesting result is the production of filaments in the *recA* derivative by ciprofloxacin and trimethoprim treatments. In particular, the production of filaments by these antibiotics was believed to be caused by the SOS response, which was mediated by RecA and LexA. This response induces the transcription of the *sulA* (*sfiA*) gene [Peterson et al., 1985, Kunz and Kohalmi, 1991]. SulA interacts reversibly with the protein FtsZ causing the inhibition of cell division and the consequent filamentation [Huisman and D'Ari, 1981]. A *sulA*-independent filamentation is also discovered in *E. coli*, the mechanism of which is also dependent on SOS induction [Kawarai et al., 2004]. Our results suggest that a new SOS-independent mechanism mediates filamentation in the absence of RecA. In fact, we have discovered that, at least for ciprofloxacin, filamentation occurs in a *sulA*-deficient background (not shown).

Moreover, we show that a number of antibiotics can increase genetic variation by

the stimulation of mutagenesis in the treated bacteria, suggesting that antibiotic treatments may favor the acquisition and/or the evolution of antibiotic resistance in bacteria. For instance, some extended-spectrum β-lactamases are the result of combining a reduced number of mutations [Morosini et al., 1996, Morosini et al., 1998]. Thus, sub-lethal concentrations of mutagenic antimicrobials (not necessarily β-lactams) may accelerate the evolution of new extended-spectrum variants by stimulating the production and the accumulation of mutations. Another example is the resistance conferred by the increased expression of efflux pumps. It can occur via mutation in different targets, including mutations in the local repressor gene, mutation in a non-related global regulatory gene and changes in the promoter region of the efflux-pump gene (see for instance reference [Piddock, 2006]).

The stimulatory effect on mutagenesis described in this work for some of the tested antimicrobials is very low, and may be considered too modest to exert any effect on bacterial evolution. However, it has been stated that modest changes in mutation rate can influence antibiotic resistance developments greatly [Denamur et al., 2005]. Concerning the possibility of finding the stimulatory concentrations by a sufficiently dense bacterial population, we have studied a vast amount of bacteria challenged by antibiotic treatments. Antibiotics are mainly used to combat pathogens but they also challenge commensals collaterally. While an infection is usually produced by a relatively small number of cells (10^8-10^9), about 10^{14} prokaryotic cells from hundreds of different species conform our commensal flora with different intrinsic levels of antibiotic susceptibility [Andremont, 2003]. Finally, even resistant microorganisms might be included among the possible targets for the mutagenic effect of antibiotics as high concentrations of antibiotics must be considered sub-lethal for resistant bacteria. Thus, any particular window of sub-MIC mutation-stimulating concentrations of antibiotics should not be difficult to find. The fact that thousand of tons of antibiotics are used every year to treat billions of human and veterinary infections and to promote animal growth, increases the probability of finding the suitable conditions for the stimulation of mutagenesis.

Studies by Romesberg and coworkers have shown that prevention of SOS acti-

vation resulted in decrease of both survival and mutagenesis in ciprofloxacin-treated cultures, as well as ciprofloxacin or rifampicin-treated infected mice [Cirz et al., 2005, Cirz and Romesberg, 2006]. Consequently, the possibility that components of induced mutation pathways might be inhibited as a novel therapeutic strategy to prevent the development of antibiotic resistance has been proposed in the literature [Cirz and Romesberg, 2007]. Efforts have been made to identify small molecules and short peptide inhibitors of RecA activity, although the absence of potential adverse effects on Rad51 (the human RecA homologue) needs to be demonstrated [Wigle and Singleton, 2007, Wigle et al., 2009].

Our study aimed at exploring the effect of RecA inhibition on the induced mutagenesis produced by many antibiotics. Our results with the *recA*-defective strain suggest that most, if not all, mutagenesis induced by sublethal concentrations of antibiotics is dependent, directly or indirectly, on RecA activity, thus supporting the hypothesis that inhibition of RecA is a plausible therapeutic adjuvant in combined therapy to reduce the capacity for generating antibiotic resistant mutants, with additional advantages of affecting sensitivity, homologous recombination [Kowalczykowski et al., 1994], swarming motility, [Gomez-Gomez et al., 2007] and biofilms [Boles et al., 2004].

5.2 Antibiotic resistance of *M. smegmatis*

In this study, we tried to identify the mechanisms which could provide acquired antibiotic resistance of mycobacteria. We analyzed a *M. smegmatis* mutant library for rifampicin resistance. We found 16 transposon mutants that increased the resistance of *M. smegmatis* to rifampicin from our insertion mutant library. Among them, we studied two independent insertion mutants carried insertions within the same target gene, *trkA* ($MSMEG\_2771$), predicted to encode a regulator of K^+ uptake and one mutant having insertion in the *ich* gene predicted to encode a K^+ transporter. The level of antibiotic resistance was identical (16-fold increase) in the $\triangle trkA$ in-frame deletion mutant, which was complemented by the wild-type *trkA* gene. The $\triangle ich$ in-frame deletion mutant also has a high level of rifampicin resistance (16 fold increase).

This mutant then was complemented by the *ich* gene of the wild-type strain.

5.2.1 TrkA

Our results show that TrkA is necessary for K^+ uptake in *M. smegmatis*, in accordance with studies that indicate it is essential for K^+ accumulation in prokaryotes, which need a high intracellular K^+ concentration (0.1-1 M) for survival [Dosch et al., 1991, Parra-Lopez et al., 1994, Chen et al., 2004, Nakamura et al., 1998]. Here, we show that the *M. smegmatis trkA* mutant requires an additional K^+ supply to counterbalance its growth defect and its susceptibility to acidic pH. Given the K^+ deficiency in $\triangle trkA$ cells, we suggest that TrkA-dependent ion acquisition is central to the maintenance of adequate K^+ levels in *M. smegmatis*.

In bacteria, proteins involved in basic physiological functions have an essential role in intrinsic resistance to antibiotics and the acquisition of antibiotic resistance [Fajardo et al., 2008, Liu et al., 2010]. Here, we demonstrate that in addition to increased rifampicin resistance, the *M. smegmatis* $\triangle trkA$ mutant exhibits enhanced isoniazid susceptibility. Interestingly, overexpression of CeoB, the *M. tuberculosis* TrkA homologue, confers isoniazid resistance in an *E. coli oxy R* mutant [Chen and Bishai, 1998]. This effect was suggested to be due to TrkA sequestration of isoniazid, as it is chemically similar to the NAD^+ nucleotide. 35 This hypothesis is supported by a recent study in which TrkA showed high affinity for active isoniazid adducts in complex with $NAD(P)^+$ [Argyrou et al., 2006]. Another group suggests that flavin adenine dinucleotide-binding proteins also influence $NADH/NAD^+$ levels, and, hence, modify isoniazid activation and binding to its main target, InhA, through nucleotide competition [Miesel et al., 1998, Vilcheze et al., 2005]. Our results indicate that the mycobacterial protein TrkA protects cells from the first-line antibiotic isoniazid, increasing tolerance of this drug by *M. smegmatis*. In addition to the previously proposed mechanisms, our results suggest that the chemical properties of isoniazid (high solubility and polarity) influence its penetration inside *M. smegmatis* and, thus, it could contribute to explain why the *trkA* mutant is more susceptible (see below).

TrkA is necessary for intrinsic resistance to a number of positively charged antibiotics in prokaryotes, such as polycationic antimicrobial peptides in *E. coli*, *Salmonella enterica* and *Vibrio vulnificus* [Parra-Lopez et al., 1994, Chen et al., 2004] or aminoglycosides in *Pseudomonas aeruginosa* [Lee et al., 2009]. We found that loss of TrkA activity modifies *M. smegmatis* susceptibility to a wide variety of antibiotics. The *M. smegmatis* $\triangle trkA$ mutant showed hypersusceptibility to cationic agents, such as aminoglycosides, and increased resistance to large hydrophobic antibiotics, including rifampicin, novobiocin and fluoroquinolones. In *E. coli*, Trk proteins, including TrkA, are also associated with a general function in antibiotic susceptibility [Girgis et al., 2009]. Disruption of the Trk system increased antibiotic tolerance when *E. coli* cells were exposed to sublethal concentrations of several classes of antibiotics with different targets (nalidixic acid, piperacillin, tetracycline and doxytetracycline), although the molecular basis of this effect was not identified [Girgis et al., 2009]. K^+ uptake via the TrkA system has a major role in bacterial physiology [Epstein, 2003, Su et al., 2009] as it has a function in osmotic stress tolerance, internal pH maintenance, the regulation of protein activity and the control of bacterial virulence. *M. smegmatis* maintains intracellular pH near neutral and a constant PMF through interconversion of the membrane electrical potential to a pH gradient.

Here, we show that TrkA activity counteracts the effect of extracellular acidic pH in *M. smegmatis* and, hence, participates in controlling intracellular ionic balance. Our results strongly suggest that in the absence of TrkA-dependent K^+ uptake, pH homeostasis fails and intracellular pH decreases to lethal levels in acidic conditions. Furthermore, it has been demonstrated that pH could affect K^+ flux and regulate the activity of K^+ uptake systems in prokaryotes [Epstein, 2003]. Therefore, the effect of pH on K^+ transport in *M. smegmatis* needs to be explored in detail.

Although a connection between TrkA-dependent K^+ transport and cell physiology has been established in prokaryotes, its association with antibiotic tolerance remains to be analyzed in detail. General alterations in antibiotic susceptibility are frequently caused by permeability changes in the mycobacterial cell envelope due to electrostatic perturbations [Nguyen and Thompson, 2006]. Our results suggest that

the mechanism by which TrkA controls multidrug susceptibility is similarly based on regulation of K^+ transport, and its effect on pH homeostasis and membrane permeability. K^+ uptake is essential for the maintenance of a constant PMF through the interconversion of membrane electrical potential to a pH gradient in Actinobacteria [Follmann et al., 2009]. Impaired K^+ uptake by inactivation of the Trk system or K^+ insufficiency increase membrane potential in *E. coli* [Bakker and Mangerich, 1981]. In the yeast Schizosaccharomyces pombe, changes in membrane potential are associated with pleiotropic changes in the susceptibility to chemotherapeutic agents due to impaired K^+ uptake [Thornton et al., 2005]. Here, we demonstrate that the lack of TrkA-dependent K^+ uptake in *M. smegmatis* leads to increased membrane potential. The hyperpolarized membrane of the *M. smegmatis trkA* mutant could attract and facilitate the penetration of positively charged antibiotic molecules into bacterial cells, but might also reduce the diffusion of large hydrophobic drugs, such as rifampicin or novobiocin. Additionally, both membrane potential and intracellular pH are key components that control the activity of PMF-dependent multidrug efflux pumps in prokaryotes [Su et al., 2006, Eicher et al., 2009]. Therefore, an impaired K^+ uptake could also indirectly modify the activity of efflux pumps in *M. smegmatis* and may influence the susceptibility to antibiotics. However, our results showed a correlation between the physicochemical properties of the antibiotics with their activities against *M. smegmatis*, suggesting a direct effect of K^+ uptake rates on bacterial permeability to antimycobacterial agents.

The acquisition of rifampicin resistance in *M. smegmatis* by *trkA* inactivation entails a conspicuous growth defect and a clear loss of fitness, probably due to the impact on cell physiology of ionic imbalance and membrane hyperpolarization. In addition, *trkA* mutants are attenuated for virulence in several bacterial pathogens, including *M. tuberculosis* [Parra-Lopez et al., 1994, Chen et al., 2004, Rengarajan et al., 2005] suggesting a low frequency of *trkA* (*ceoB*) mutations among rifampicin resistant *M. tuberculosis* clinical isolates; however, the occurrence of different *trkA* mutations in *M. tuberculosis* strains remains to be explored. Our results indicate that by modifying cell permeability, alterations in ion transport promote a change in the *M. smegma-*

tis susceptibility pattern to antibiotics. K^+ supply might be an important element in this effect; variations in K^+ levels could influence antibiotic diffusion and produce changes in drug susceptibility. In addition to *trkA*, the *M. smegmatis* genome contains two others genes, $MSMEG\_2769$ (*trkB*) and $MSMEG\_1945$ (*ich*), which encode a putative K^+ transporter (see below). Mycobacteria also encode another main K^+-uptake system, named Kdp, a P-type ATPase. Kdp is an inducible system with high affinity for K^+ and requires ATP hydrolysis to promote K^+ uptake in *E. coli* [Greie and Altendorf, 2007]. Furthermore, ABC components could also develop a regulatory role in Trk-mediated K^+ uptake [Harms et al., 2001]. As other proteins involved in ion transport might also influence antibiotic effectiveness, it would be of interest to explore the role of ion transporters in intrinsic resistance and the acquisition of drug resistance in mycobacteria. On the other hand, K^+ uptake is inhibited by some compounds with anti-mycobacterial activity, such as the riminophenazine clofazimine [Steel et al., 1999]. Our data, nonetheless, indicate that the inhibition of K^+ uptake could induce a complex pattern of phenotypes, including increased resistance to anti-mycobacterial drugs such as rifampicin. Such collateral resistance should be carefully considered when combined treatments are designed.

5.2.2 Ich

We found that the $\triangle ich$ mutant need an additional amount of K^+ to grow. Indeed, it grew better when more K^+ was added to the medium at different concentrations. This result provides physiological evidences that the Ich protein may function as a potassium transporter. Our study also suggested that the role of the Ich protein in potassium transport relies on its structure which contains the TrkA-N domain having a nucleotide-binding sequence previously identified as a flavin adenine dinucleotide-binding motif.

While the mutants lacking of the *ich* gene poorly grew in normal MB medium, their growth was restored by adding more K^+. The fact that additional K^+ are required for the mutants lacking of *ich* to balance their growth suggests that the Ich protein may play an important role in the maintenance of sufficient K^+ concentration

in the cells of *M. smegmatis*.

In general, ionic channels are very important in underpinning numerous fundamental physiological processes. Therefore, ionic channels are certainly considered as a major class of drug targets [Clare, 2010]. In bacteria, this kind of proteins plays an important role in antibiotic resistance. For instance, the K^+ uptake system Trk including TrkA was determined to be associated with general functions in antibiotic resistance profile in many bacteria such as *E. coli* [Girgis et al., 2009] and *Pseudomonas aeruginosa* [Lee et al., 2009].

Our experimental results showed the participation of the *ich* gene having a TrkA domain in antibiotic resistance profile of *M. smegmatis*. On one hand, we found that the deletion of the *ich* gene in *M. smegmatis* was associated with hypersusceptibility to hydrophilic antibiotics (cationic agents) such as INH and aminoglycosides. Yet on the other hand, it was also associated with the increasing resistance to many other hydrophobic/lipophilic antibiotics such as rifampicin, novobiocin and floroquinolones.

In addition to the decrease of the susceptibility to rifampicin, the mutants lacking of *ich* increase the susceptibility to INH. This result demonstrated the role of *ich* in the protection of *M. smegmatis* from INH. Because the Ich protein has a TrkA-N domain, the Ich protein can protect bacteria from INH based on those two previous mechanisms described in the TrkA part (see above). Furthermore, the double mutant with deletions of both genes *trkA* and *ich* showed higher susceptibility to INH. As the $\triangle trkA$ mutant of *M. smegmatis* was determined to increase its susceptibility to INH in our previous work [Castaneda-G'arcia et al., 2011], we hypothesize that the deficiency of both genes causes the increasing of susceptibility to INH.

An increase of susceptibility to ethionamide was observed in mutants lacking of the *ich* gene and the double mutant with deletions of both genes *ich* and *trkA*. Ethionamide and INH have a similar structure and the mechanism of both drugs requires the activation of prodrug [Johnsson et al., 1995]. Although there are significant differences in their activation mechanisms, both of these drugs have a common target, i.e. InhA. It has been determined that the alteration of $NADH/NAD^+$ ratios is not only related to the activation of INH as we have discussed above but also involved

in the activation of ethionamide. Indeed, the change of the NADH/NAD^+ ratios is associated with inhibiting the binding of INH and ethionamide to their target InhA [Vilcheze et al., 2005]. Interestingly, we did not see the increase of susceptibility to ethionamide of the mutant lacking of *trkA* via identification of its MIC. Another interesting result is that, the $\triangle ich$ mutant is more susceptible to INH than $\triangle trkA$. Therefore, we hypothesize that the change of NADH/NAD^+ ratios maybe much larger in $\triangle ich$ than that in $\triangle trkA$. This hypothesis should be verified more carefully by experimental works in the future.

In the literature, it has been determined that the presence of K^+ is necessary for the maintenance of neutral internal pH in several well-known bacteria such as *E. coli* [Kroll and Booth, 1981], *Lactococcus lactis* [Kashket and Barker, 1977] and also *Steptococcus mutans* [Dashper and Reynolds, 1992]. Experiments about the growth of bacteria at pH value ranging from 5 to 8 in the absence of K^+ and at pH value 5.5 in the presence of K^+ confirmed the importance of K^+ under acidic conditions. An increase of sensitivity to acidic conditions was observed with mutants without the *ich* gene. Indeed, the mutants were growing with similar to growing level of wild-type at alkaline and neutral pH, but their growth was significantly impaired at low pH values. At pH 5.5, additional K^+ was necessary to support the growths of mutants. In the presence of sufficient K^+ (100mM), the $\triangle ich$ mutant almost reach the growth level of wild-type. Interestingly, we found that the double mutant with deletions of both genes *ich* and *trkA* had almost lost its capacity at low pH (5.5). Besides, when the single mutants have recovered their growth with the additional K^+, the growth of double mutant was still very bad.

Our results demonstrate that the disruption of K^+ transport by the loss of Ich function impaired the capacity for pH homeostasis in *M. smegmatis*, and the intracellular pH decreases significantly to lethal level in acidic conditions. Therefore, Ich plays an important role in the growth at acidic conditions. Moreover, the internal pH is also regulated by the other system such as the K^+ efflux and the K^+ uptake systems. Particularly, our results together with the results of our previous work indicated that both Ich and TrkA participated in the regulation of internal pH. However,

the role of the *ich* gene in the regulation of internal pH is still unveiled and need more efforts to understand it deeply.

We found that mutants with inactivation of Ich protein showed an increase of $\triangle\psi$ which were then decreased by the adding K^+. The highest membrane potential was observed in the double mutant lacking of two genes *trkA* and *ich*. Because both of these genes involve in the regulation of membrane potential via the K^+ transport, their absence should cause the highest unbalance in the K^+ transport. As a consequence, it causes the highest difference between concentrations of K^+ inside and outside the cell. It is also a reason of the observation that the highest membrane potential was obtained in double mutant cells.

The ionic imbalance and hyperpolirization may lower the growth of the mutants with the *ich* deletion. Moreover, we suggested that the modification of membrane permeability, the change in ionic transport of *M. smegmatis* take a crucial part in antibiotic resistance profile. The supplement of K^+ could be considered as an important factor in this process. Indeed, the maintained K^+ inside the cells may have an effect in the penetration of antibiotics across the membrane and the change in K^+ transport. Thus, it can cause alternations in antibiotic susceptibility. In this work, we determined the function of the Ich protein as a putative K^+ channel. Furthermore, the genome of *M. smegmatis* also has other proteins taking part in the K^+ transport including the uptake the efflux systems. It would be interesting to study the role of ionic transporters in the resistance development to antibiotics in mycobacteria. As we have discussed above, the membrane potential and intracellular pH are important factors which regulated the activity of the drug efflux pumps depending on PMF in prokaryote [Su et al., 2006, Eicher et al., 2009]. Thus, the K^+ transport could have effects on the function of drug efflux pumps. The relation between the K^+ transport system, in particular the activity of the Ich protein, and the function of drug efflux pumps should be carried in a future work to identify the molecular mechanisms of drug resistance in mycobacteria.

Chapter 6

Conclusions

1. Sublethal concentrations of eight antibiotics, including ampicillin, ceftazidime, trimethoprim/sulfamethoxazole, imipenem, ciprofloxacin, trimethoprim, sulfamethoxazole, and tetracycline, are able to stimulate mutagenesis of *E. coli*.

2. While most antibiotics have a slight effect with mild increase in mutagenesis, trimethoprim, sulfamethoxazole and the combination of trimethoprim plus sulfamethoxazole produced the highest effect with highest increase in mutant frequency for both rifampicin and fosfomycin resistance. In this case, both SOS response and nucleotide imbalance might act synergistically to increase mutant frequency.

3. Inactivation of *recA* abolishes the induction of mutagenesis produced by different antibiotics. Thus, most mutagenesis induced by sublethal concentrations of antibiotics depends on RecA activity.

4. The disruptions of *trkA* and *ich* result in the increase of rifampicin resistance in *M. smegmatis*.

5. TrkA mediates K^+ uptake, and it is essential for maintaining the growth of *M. smegmatis*, its pH homeostasis and membrane potential.

6. The Ich protein is a putative K^+ transporter which plays an important role in the maintenance of sufficient K^+ concentration in the cells of *M. smegmatis*.

Similar to TrkA, Ich is essential for the growth at acidic conditions, it also takes part in the regulation of membrane potential of bacteria.

7. TrkA takes an important part in regulation of antibiotic susceptibility. Deletion of the *trkA* gene modifies *M. smegmatis* susceptibility to a wide variety of antibiotics. Indeed, the *M. smegmatis* $\triangle trkA$ mutant showed hypersusceptibility to cationic agents, such as aminoglycosides, and increased resistance to large hydrophobic antibiotics, including rifampicin, novobiocin and fluoroquinolones.

8. Inactivation of ich also causes the change of antibiotic resistance profile of *M. smegmatis*, mutants lacking of *ich*, on one hand, become resistance to hydrophobic antibiotics such as novobiocin and rifampicin, and on the other hand, they are more susceptible to hydrophilic antibiotics, such as isoniazid and aminoglycosides

9. TrkA and Ich are considered as general regulators of antibiotic susceptibility. The change in antibiotic resistance profile of *M. smegmatis* in the absence of the TrkA or Ich proteins is associated with membrane hyperpolarization.

Chapter 7

List of journal articles published during the thesis work:

1. Thi Thuy Do, Elena López, Alexandro Rodríguez-Rojas, Jerónimo Rodríguez-Beltrán, Alejandro Couce, Javier R. Guelfo, Alfredo Castaneda-García, and Jesús Blázquez. 2011. "Effect of *recA* Inactivation on Mutagenesis of *Escherichia Coli* Exposed to Sublethal Concentrations of Antimicrobials." The Journal of Antimicrobial Chemotherapy 66 (January 5), Oxford University Press.

2. Castaneda-García, Alfredo, Thi Thuy Do, and Jesús Blázquez. 2011. "The K^+ Uptake Regulator TrkA Controls Membrane Potential, pH Homeostasis and Multidrug Susceptibility in *Mycobacterium Smegmatis*." The Journal of Antimicrobial Chemotherapy 66 (7) (July), Oxford University Press.

List of Figures

List of Tables

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