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Structural characterization of bacterial proteins involved in antibiotic resistance and peptidoglycan biosynthesis

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1. Introduction

"The time may come when penicillin can be bought by anyone in the shops.
Then there is the danger that the ignorant man may easily underdose himself
and by exposing his microbes to non-lethal quantities of the drug make
them resistant. Here is a hypothetical illustration. Mr. X. has a sore throat.
He buys some penicillin and gives himself, not enough to kill the streptococci
but enough to educate them to resist penicillin. He then infects his wife.
Mrs. X gets pneumonia and is treated with penicillin.
As the streptococci are now resistant to penicillin the treatment fails. Mrs. X dies.
Who is primarily responsible for Mrs. X's death?
Why Mr. X whose negligent use of penicillin changed the nature
of the microbe. Moral: If you use penicillin, use enough."

Alexander Fleming, Noble lecture, 1945

Antibiotic discovery and tuberculosis (TB) interwove tightly during the course of history, with TB posing some of the most difficult challenges for scientists and physicians working in the field of human healthcare. TB is among the most ancient diseases that ever afflicted the human species. It caused and still causes millions of deaths every year, and treatments are still not always effective. Even after penicillin was discovered in the early 1930's, TB remained an uncured disease, and a decade had to go by before an anti-TB antibiotic was found. Antibiotics are one of the most significant medical achievements of the 20th century, as they enabled the success of modern medicine by dramatically improving the outcome of surgical operations, and allowing the treatment of bacterial infections in immunocompromised patients, like, for example, elderly people, cancer and HIV-positive patients, and diabetics [124,279]. However, the effectiveness of antibiotics is constantly challenged by the evolution of bacterial resistance, and again, TB is a prototypical example of this phenomenon. Here, the past and ongoing history of TB and antibiotic discovery will be presented, and possible future perspective will be discussed.

A world-wide threat: tuberculosis

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (Mtb). TB is responsible for millions of deaths every year, and in 1993, it was declared a global emergency by the World Health Organization (WHO). Although joined efforts by the WHO and the United Nations (UN) managed to halve TB mortality between 1990 and 2015, and reduce its prevalence by 42%, TB still figures as one of the top ten causes of death in 2015, ranking above HIV/AIDS as a deadly infectious disease. It was estimated that TB infected 10.4 million new people in 2015, and caused the death of 1.4 million people, while another 0.4 million died of TB among the HIV-infected population [274]. Thus, the WHO and

the UN established new goals for the period 2016-2035 with the Sustainable Development Goals (SDGs) and the End TB Strategy, respectively, which aim at a 95% reduction of TB mortality and a 90% reduction of TB incidence by 2035 compared to 2015 [274].

Stages of TB infection

TB infection spreads through air droplets that contain the bacilli. After the air droplets are inhaled and reach the alveoli in the lungs, bacteria adhere to the tissue surface and start dividing. The alveolar macrophages of the host immune system react against the infection by surrounding and swallowing the bacteria. However, bacteria are not destroyed but remain alive within the macrophages, which form granulomas, where infected macrophages are surrounded by other non-infected cells of the immune system. If the host immune system is strong, it succeeds in containing all bacteria, in which case the TB infection becomes inactive or latent. The patient would test positive for TB but would not be contagious, because the TB bacilli are isolated from the lungs and cannot enter the airways. The infection can remain latent for a long time, even for the entire life of the infected person. In healthy individuals, granulomas can heal, leaving a sort of calcified scar that is visible by X-ray. However, if the host immune system is weak, like in elderly and malnourished people or immunocompromised patients, the TB infection can reactivate and TB bacilli start reproducing inside the macrophages, ultimately breaking them open. When the infection gets reactivated, exponential bacterial division occurs and macrophages are not able to take up all new TB bacilli, and the patient develops active pulmonary tuberculosis. At this stage, TB symptoms like coughing and fatigue start to manifest, and the infected person is highly contagious because his sputum contains active TB. In the worst cases, the infection can escape the lungs and enter the blood stream, leading to extrapulmonary TB infection.

TB in history

TB is the archetype of human pathogens, having evolved with the human race [48]. The finding of human fossil bones, mainly vertebrae, with clear signs of bone TB allowed to date TB infections back to about 8000 BC [105], and Mtb DNA could be amplified from tissue samples of an Egyptian mummy from the New Kingdom (1550-1080 BC) presenting bone deformities and a collapsed lung [45,192]. Even though the etiology of TB remained unknown to men for millennia, many testimonies of TB are found throughout history. The first written document reporting about human TB is attributed to the Babylonian monarch Hammurabi between 1948 and 1905 BC, and later, in 1500 BC, descriptions of TB as consequence of fatigue, worries, pregnancy, hunger, and chest wounds appeared in Indian literature [105]. The words *phthisis* and *consumption*, that remained the

most used names for the disease until the end of the 18th century, were first introduced in Ancient Greece by Hippocrates (460-370 BC), who described typical symptoms of TB and recognized its different entity compared to other pulmonary diseases [46]. TB also appears throughout Roman literature, and in texts from the Middle Age and the Renaissance. In those times, however, phthisis, consumption, scrofula, and other forms of TB were regarded as different diseases. In the 17th century, thanks to the introduction of biopsy on human bodies, the Dutch physician and scientist Franciscus de la Boë Sylvius (1614-1672) correlated for the first time the presence of tubercles in the lungs and organs with consumption, and that of phthisis with the skin condition of scrofula [105]. The definite unification of the different forms of TB, whether pulmonary or extrapulmonary, was made in 1819 by the French physician René Théophile Hyacinthe Laennec (1781-1826), who fathered with his treatise the modern understanding of TB, introducing medical terms for describing the pathology that are still in use today [45]. In 1834, Johann Lukas Schönlein of Würzburg (1793-1864) introduced the word *tuberculosis* to describe affliction with tubercles, and together with several other notable scientists of the time, like Friedrich Gustav Jakob Henle (1809-1885) and Theodor Albrecht Edwin Klebs (1834-1913), attempted to elucidate the nature of the fundamental cause of TB. In the 18th century, scientists in Northern Europe tended to regard TB as a hereditary condition, while in Southern Europe the disease was considered communicable. Notably, the first to believe in the communicability of TB was Aristotle (384-322 BC) in Ancient Greece, and later in Italy, Fracastorius postulated that the phthisis was transmitted through an invisible “virus” that could survive in the clothes of consumptives up to two years. That the objects of consumptives were of danger for human health was also stated in an edict issued by the Republic of Lucca in 1699. Another Italian physician, Giovanni Battista Morgagni of Padua (1682-1771), was also convinced that consumption was contagious, as shown by his refusal to perform post-mortem examinations on consumptives for fear of infection [105]. In 1720, the English physician Benjamin Marten (1690-1752) postulated the idea that consumption could be caused by “certain species of *Animalcula* or wonderfully minute living creatures” that could be possibly “carried about by the air” and deposited in the lungs [54]. In 1877, Theodor Klebs succeeded in keeping the causative agent of TB alive on a protein medium and showed that it could induce TB in animals. However, the definitive proof that TB is caused by microbes came from the German scientist Robert Koch (1843-1910). In a memorable lecture at the Physiological Society in Berlin in 1881, and later in his paper *Die Ätiologie der Tuberkulose* (1882), Koch showed some rod-shaped bacteria that he had visualized in the tuberculous tissue using a new staining method and which he called tubercle bacilli. The tubercle bacilli were later renamed *Mycobacterium tuberculosis*. Koch, who had already been working on isolating bacteria in pure cultures, also showed that pure isolated colonies of the

tubercle bacilli could induce TB in healthy guinea pigs [24]. It is worth mentioning that at the same time another German scientist, Paul Clemens von Baumgarten (1848-1928), also visualized *M. tuberculosis*, but his work was published shortly after Koch's lecture to the Physiological Society [105]. However, elucidation of the etiology of TB still did not provide a cure for the disease, and Koch's attempts to cure TB by tuberculin, a glycerin extract of dead tubercle bacilli, proved ineffective against the disease. Even if tuberculin failed as a cure, it became a fast screening method for diagnosis of TB infections. Tuberculin was applied under the top layer of skin on the inner forearm, and caused a cutaneous rash only in people infected by TB [222]. "For his investigations and discoveries in relation to tuberculosis", Koch was awarded the Noble Prize in Medicine or Physiology in 1905.

TB treatment in the pre-antibiotics era

That rest and fresh air could help in handling TB was already known under the Roman Emperor Marcus Aurelius in 174, as attested by the work of the Greek physician Galen, who recommended fresh air, milk, and sea voyages to consumptives [45]. For a long time, rest and fresh air remained the only remedies, and in the 19th century, the German physician Herman Brehmer (1826-1889) opened the first sanatorium for the treatment of consumptives. Brehmer's idea was that by transferring consumptives to a TB-immune place, where no cases of TB were reported, patients would heal by themselves [105]. Although his idea was proven wrong, many other sanatoria were opened in Europe and in the USA on the model of Brehmer's first sanatorium. Treatment in sanatoria worked in some cases, and in some other managed to stabilize or improve the condition of the patient. On the long term however, patients often relapsed and eventually died of TB. In addition to rest in sanatoria, artificial pneumothorax became a common TB treatment towards the end of the 19th century. The first observation that pneumothorax could cure pulmonary TB dates back to 1696, when the Italian doctor Giorgio Baglivi (1668-1707) reported a tuberculous patient who healed after incurring pneumothorax as a consequence of a sword wound. In 1834, F. H. Ramadge executed the first therapeutic pneumothorax in London, and in 1888, the Italian physician Carlo Forlanini (1847-1918) created the first artificial pneumothorax [228], making pneumothorax a safer technique. In some cases, partial unilateral rib resection, or thoracoplasty, could be executed with the aim of reducing the thoracic cavity and causing the collapse of tubercular cavities [105].

Attempts to control TB by vaccination were also made long before the discovery of any medicine against TB. Frenchmen Léon Charles Albert Calmette (1863-1933) and Jean-Marie-Camille Guérin (1872-1961) developed a vaccine by attenuation of a strain of *M. bovis* that was called the bacillus Calmette-Guérin (BCG) vaccine. Already in 1921 in Paris, the first vaccinations started using the BCG vaccine, and still today it is the only available vaccine against TB. The BCG

vaccine was proven to be 80% effective in protecting healthy children from the most aggressive forms of tuberculous meningitis and miliary TB (TB widespread through the body), but it offers little protection to adults from the deadliest pulmonary TB [50]. Because the BCG vaccine consists of a live bacterium even if weakened, it cannot be administered to HIV-positive people, because the vaccine itself would cause the disease in these patients [105].

The antibiotic era

In 1928, Sir Alexander Fleming (1881-1955), a bacteriologist at the St. Mary's Hospital in London, noticed a halo of bacterial lysis around a colony of mould that had contaminated a culture plate of staphylococci. Although Fleming was not working on antimicrobials at the time, he went on to isolate the mould to purity and characterize its antibiotic activity [80,81]. The mould was identified as the penicillin-producing *Penicillium notatum*, and penicillin was the molecule with antibiotic activity, which is exerted by binding of penicillin to penicillin-binding proteins (PBPs), thus interfering with the biosynthesis of peptidoglycan. Penicillin was later developed into a drug by two other scientists, Howard Walter Florey (1898-1968) and Ernst Boris Chain (1906-1979), who implemented the large-scale production of penicillin at the beginning of 1940's [31]. In 1945, Fleming, Florey and Chain were jointly awarded the Noble Prize in Physiology or Medicine "for the discovery of penicillin and its curative effect in various infectious diseases". The discovery of penicillin completely revolutionized the treatment of bacterial infections but, nevertheless, TB remained uncured due to the inherent resistance of Mtb to penicillins. The knowledge that bacteria are able of inhibiting the growth of other microbes was already present in the late 80's of the 19th century. In Italy in 1885, the physician Arnaldo Cantani (1837-1893) realized that pulmonary TB could be treated with inhalations of pure liquid cultures of *Bacterium termo*, a common saprophytic bacterium, and in 1888, Victor Babeș (1854-1926) in Romania showed that the metabolites of saprophytic microorganisms such as *Bacterium prodigiosum* and staphylococci could inhibit growth of tubercle bacilli. However, a systematic study of the inhibitory effects that bacteria and fungi exerted on each other was still missing. First as a student at the University of Berkley, California, and later as a professor at Rutgers University, Selman Abraham Waksman (1888-1973) observed the potent growth inhibitory activity that actinomycetes had on other bacteria. During the years, Waksman gathered a collection of over 500 *Streptomyces* strains, and started testing these soil bacteria in search of anti-TB activity. His screening technique is often referred to as the Waksman platform for antibiotic discovery [40]. The first two antibiotics that Waksman found by his platform, actinomycin and streptothricin, could not be used in the clinic because of their high toxicity in humans. In 1943, however, a new antibiotic called streptomycin was isolated from *Streptomyces griseus* by Albert Israel Schatz

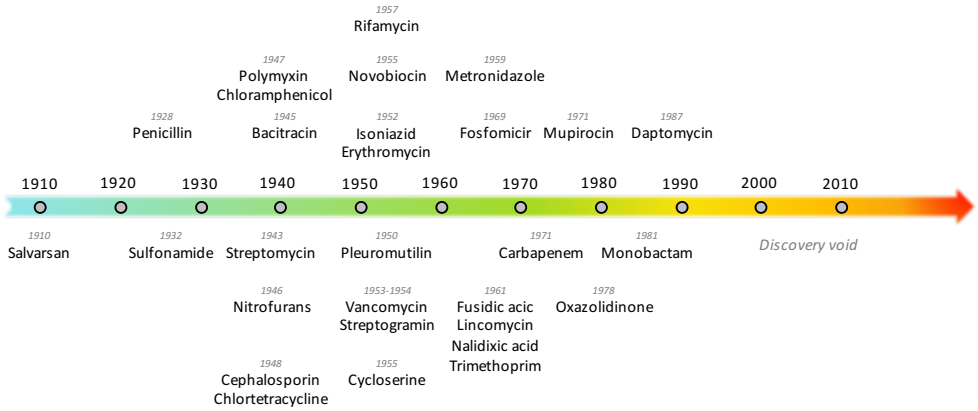

Figure 1. *Discovery timeline of antibiotic classes (adapted from* [273]*).*

(1920-2005), a PhD student in the laboratory of Prof. Waksman. Streptomycin is an aminoglycoside antibiotic that inhibits the synthesis of bacterial proteins. In collaboration with Merck and the Mayo clinic, the first clinical trials were done in 1946, and despite several side effects, including loss of hearing and/or balance, streptomycin allowed to fully cure severely ill TB patients for the first time. However, after a few months from the introduction of the antibiotic into the clinic, doctors had to face the appearance of resistance to streptomycin [105,291]. Already in his Nobel Prize acceptance speech in 1945, Fleming had warned the scientific community of the ability of bacteria to develop resistance to antibiotics, rendering them ineffective in curing diseases [80]. The problem of antimicrobial resistance (AMR) was easily tackled during the so-called Golden Age of antibiotic discovery in between 1940's and 1980's, thanks to the Waksman platform, which allowed a continuous pipeline of antibiotic discovery, with twenty different classes of antibiotics being identified [155] (Fig. 1). Among them, another antibiotic class active against TB, the rifamycins, was discovered by Prof. Piero Sensi (1920-2013) and Dr. Maria Teresa Timbal (1925-1969) at Lepetit Pharmaceuticals in Milano. By analyzing a soil sample collected in a pine forest close to the small city of Saint-Raphaël on the French Riviera, the researchers found a new bacterial species, *Amycolatopsis rifamycinica*, which was producing several molecules with antibiotic activity. These molecules were called rifamycins, from the name of the movie Rififi by which the two microbiologists used to call the soil sample coming from Saint-Raphaël. The first member of the rifamycins was the antibiotic rifampicin, which was introduced in the clinics in 1966, and was destined to be the last developed anti-TB drug for the next fifty years [218]. Rifamycins inhibit the bacterial RNA polymerase, and besides providing a cure to TB, also proved effective against leprosy and complex infections by *Mycobacterium avium*. However, as it had

already happened for streptomycin, rifampicin monotherapy soon selected for antibiotic resistant Mtb [43,165].

Combination therapies

The ability of readily developing resistance to chemotherapy is now a well-known characteristic of Mtb [174,291]. Besides being resistant to mono-chemotherapy, TB has also been reported to be multidrug resistant (MDR), i.e. resistant to at least rifampicin and isoniazid, and more recently, extensively drug-resistant (XDR), defined as MDR-TB with additional resistance to a fluoroquinolone and at least one second-line injectable agent, like amikacin, kanamycin, or capreomycin [53,291].

A strategy to more effectively treat TB, and hopefully avoid the emergence of MDR- and XDR-TB is the use of combined drug therapies [41]. The first combination therapy used in the treatment of TB was introduced before the discovery of rifampicin, and consisted of streptomycin, *para*-aminosalicylic acid (PAS), and isonicotinic hydrazide (isoniazid). PAS was developed by Jörgen Erik Lehmann (1898-1989) in Sweden in the same years as Waksman and Schatz were working on streptomycin. The idea of a modified salicylic acid molecule as anti-TB drug came to Lehmann after Dr. Frederick Bernheim from Duke University shared his results about a more than 100-fold increase in oxygen uptake by TB in the presence of salicylic acid [11]. Lehman had the intuition that an analogue to salicylic acid could have an opposite effect on Mtb, i.e. a decrease in the oxygen uptake. PAS proved Lehmann's intuition right, and showed to be effective in the treatment of TB [150]. Isoniazid is a potent anti-TB drug that acts by inhibiting the biosynthesis of mycolic acid of which the surface of Mtb is particularly rich, and it has very little toxicity in humans [237]. Isoniazid was simultaneously discovered in 1951 by Bayer in Germany, and Squibb and Hoffmann-LaRoche in the USA by screening chemicals for anti-TB activity in a similar way as Waksman had been testing soil samples. This same approach led in the following years to other chemical drugs, such as pyrazinamide, ethionamide and ethambutol [154]. After rifampicin-resistant strains were identified, new drugs were introduced for use in combination therapies, including aminoglycosides (e.g. capreomycin, viomycin, kanamycin and amikacin), fluoroquinolones (e.g. ofloxacin and ciprofloxacin), and more recently, bedaquiline and delamanid [6,15,105,235]. Guidelines from the WHO recommend combination therapies with three or more anti-TB drugs, especially in the case of MDR-TB, in order to minimize the chances of antibiotic resistance [69]. Nevertheless, although more effective in avoiding TB resistance, recent studies have highlighted the possibility that even combination therapies might lead to antibiotic resistance in Mtb [3,104,177]. The most generally recognized reason for appearance of antibiotic resistance in TB is the well-known mutability of Mtb, but also the poor adherence of patients during prolonged treatments, which usually vary between six and twenty months [69]. Additionally, it

has been recognized that the different compartments within TB lesions, and different pharmacokinetics and pharmacodynamics of each single drug also contribute to select for resistant mutants [177,209]. Thus, there is an urgent need for new anti-TB therapeutic strategies, which could be readily satisfied by one of the most common class of antibiotics, the β-lactams.

B-lactams and β-lactamases

The β-lactams, i.e. penicillin and its derivatives (e.g. penem, cephem, carbapenem and monobactam), have been one of the most widely used classes of antibiotics since their first introduction in the early 1940's [114,115]. Worldwide, it was estimated that β-lactam antibiotics account for approximately 65% of the 100,000-200,000 tonnes of antibiotics manufactured yearly [61,278]. Some of the advantages of β-lactam antibiotics are their broad spectrum of action, low toxicity for humans, favorable pharmacokinetics and pharmacodynamics, and a well-established biotechnological production that ensures their availability also in low-income countries [112]. On the down side are the resistance mechanisms to β-lactams widespread among bacteria, such as the modification of the target PBPs, or the up-regulation of drug efflux pumps activity. The most widespread mechanism of resistance, however, is the evolution of β-lactamase enzymes [20,159]. B-lactamases (E.C. 3.5.2.6.) are enzymes that inactivate β-lactam antibiotics by attacking and hydrolyzing their β-lactam rings [204]. The first β-lactamase activity, described as penicillin-inactivating mechanism, was reported as early as 1940, when penicillin was still not used in therapies [1], and during the years, more than 1400 unique β-lactamases with varying affinity for the different β-lactam substrates have been identified in microorganisms, with some β-lactamases hydrolyzing even the most potent β-lactams cephalosporins and carbapenems [48,148,152,177]. Mtb is a peculiar microorganism in that it is inherently resistant to β-lactam antibiotics because of its chromosomally encoded β-lactamase, BlaC [42].

B-lactamase classification

B-lactamases are classified according to two main systems. The Ambler classification system is based on amino acidic sequence similarity of the lactamases [4,95], and the second system on the substrates and inhibitors specificity of each enzyme [19,21,22]. The Ambler classification divides β-lactamases into four classes, A through D, solely based on their structures. Enzymes belonging to classes A, C, and D are all serine β-lactamases that hydrolyze β-lactams through an acyl-enzyme intermediate. The most common β-lactamases belong to classes A and C, like for example Ambler class A β-lactamases TEM-1 and SHV-1, or the Ambler class C AmpC [120,125]. Class B comprises metallo-β-lactamases (MBLs), which require one or two bivalent metal

ions, such as Zn^{2+}, for catalysis [21]. The Ambler classification is the most unambiguous, but it fails to provide useful clinical characteristics of β-lactamases. On the other hand, clinical aspects like affinity for different substrates and inhibition profiles are at the basis of the functional classification scheme, which identifies three groups of β-lactamases. Group 1 comprises cephalosporinases from class C, while class A and D serine β-lactamases belong to group 2, and MBLs to group 3. As a consequence of their unique structure and catalysis, MBLs are not inhibited by clavulanic acid or tazobactam, but are sensitive to inactivation with metal ion chelators, such as EDTA or dipicolinic acid [21]. Each group has got several subgroups, but the largest one is group 2, with more than ten subgroups, primarily due to the identification of a constantly increasing number of extended spectrum β-lactamases (ESBLs) triggered by the extensive use of antibiotics [173]. ESBLs not only catalyze the hydrolysis of penicillins, but also of cephalosporins, monobactams, and carbapenems, thus broadening the spectrum of bacterial resistance. The spread of new β-lactamases mostly happens through lateral gene transfer of plasmids, but *bla* genes may as well be encoded on chromosomes and integrons. When *bla* genes are transferred through integrons, they often carry resistance to other antibiotics as well, so that β-lactam resistance is often associated with multi-antimicrobial resistance [121].

B-lactam/β-lactamase inhibitors combination therapies

With the aim of not losing the therapeutic advantages offered by the β-lactam antibiotics, research has been done to find inhibitory molecules for β-lactamases, so that the use of penicillins could still be possible. Clavulanic acid (Fig. 2), produced by *Streptomyces clavuligerus*, was the first β-lactamase inhibitor discovered in the 1970's [16]. Clavulanate (the deprotonated form of clavulanic acid in solution) alone showed little antimicrobial activity, but combined with amoxicillin substantially decreased the minimum inhibitory concentrations (MICs) of the antibiotic against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Escherichia coli* [221]. Later, the penicillinate sulfones sulbactam and tazobactam were produced synthetically (Fig. 2). Clavulanate, sulbactam and tazobactam are mechanism-based inhibitors that share a similar structure to penicillin, but differ in that they have a leaving group at position C-1 of the penta-

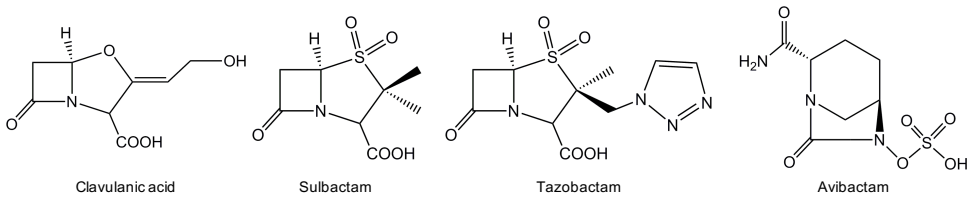

Figure 2. *Structures of the β-lactamase inhibitors approved by the Food and Drug Administration.*

membered ring. The presence of a leaving group facilitates secondary ring opening and β-lactamase inhibition by formation of a covalent adduct [57,66,140]. Newer mechanism-based inhibitors include avibactam (formerly AVE1330A or NXL104) that has been clinically approved in combination with ceftazidime and is in phase I (clinical development) in association with ceftaroline and aztreonam, and MK-7655 that is a novel β-lactamase inhibitor under investigation in combination with imipenem/cilastatin [9,23,106,153,160,208,254]. However, at the moment, clavulanate, sulbactam and tazobactam are the most used inhibitors in combination therapies in USA. Common formulations consist of amoxicillin-clavulanate, ticarcillin-clavulanate, ampicillin-sulbactam, and piperacillin-tazobactam, while in several European countries, Japan, and India cefoperazone-sulbactam is also available [57].

With the development of β-lactam/β-lactamase inhibitor concentrations, the use of β-lactams for the treatment of TB has been reconsidered, and in 1995, it was shown that indeed combinations of β-lactams/β-lactamase inhibitors were effective in killing Mtb [32]. Despite the fact that an accurate, standardized measurement of β-lactams/β-lactamase inhibitors MICs on Mtb growth is complicated by long incubation times and the intrinsic instability of many compounds in solution, all studies show that the addition of β-lactamase inhibitors improves the antibiotic activity of β-lactams [32,51,140,239]. Importantly, a recent study showed that the combination of meropenem and clavulanic acid is highly effective in treating XDR-TB [112]. However, meropenem has to be administered intravenously for a prolonged time, strongly limiting the widespread use of the meropenem/clavulanic acid combination therapy. Therefore, MDR- and XDR-TB still present major challenges, especially considering that co-resistance to β-lactams/β-lactamase inhibitors has been reported both in the lab and in clinical isolates of Gram-negative pathogens and Mtb [33,57]. Mechanisms of resistance to combination therapies include decreased cell permeability or increased efflux of drugs, overexpression of β-lactamases as a consequence of mutations in the promoter region, higher copy number of plasmids carrying the *bla* gene, and structural modification of PBPs and/or β-lactamases [57,140]. All these aspects must be taken into account when designing new combination therapies for the treatment of TB, that would also prevent the appearance of resistance mechanisms. For this reason, β-lactamase inhibitors should be systematically assessed for their role in combination therapies, their dosing and effects after prolonged treatments. The availability of several β-lactamase inhibitors to be used in combination with β-lactams gives valid hopes that such cures can be optimized in the near future [140].

Streptomycetes: a complex life cycle

Streptomycetes are common soil-dwelling, Gram-positive bacteria that are of great biotechnological interest for the production of a variety of secondary metabolites, including antibiotics, anti-fungal and anti-cancer agents, and immunosuppressants [110]. Besides their industrial relevance, streptomycetes play key environmental roles in the soil as they participate in the recycling of complex organic polymers, including cellulose, chitin, xylan, and agar [7,35,55]. *Streptomyces* grow as multicellular mycelia with a similar morphology as fungi, and follow a rather complex life cycle [7,122]. The first stage of growth consists of the germination of a single spore, which then grows out to form a vegetative hypha. The hyphae consist of long, multi-genomic cells that do not undergo binary fission, but instead are compartmentalized by irregularly spaced cross-walls. Exponential growth of vegetative hyphae is achieved by a combination of tip extension and branching [77,79]. Upon nutrient depletion, the vegetative mycelium enters a stage of programmed cell death [167] during which vegetative hyphae are digested for providing energy and building blocks for the production of air-protruding aerial hyphae. The onset of aerial growth corresponds to the onset of secondary metabolite production [13,272]. In a fully grown aerial hypha, chromosomes are condensed, segregated, and separated by regularly spaced septa to form prespores. Finally, chains of mature spores are formed [7,122].

Cell wall synthesis in Streptomyces

Genes involved in bacterial cell division were initially identified in the rod-shaped bacteria *E. coli* and *B. subtilis* by a systematic study of deletion mutants with a block of cell division but unaffected DNA replication or segregation [12]. *E. coli* and *B. subtilis* null mutants for cell division genes would grow as filaments only at permissive temperatures, so that the corresponding genes were named *fts* for filamentous temperature-sensitive phenotype [122,216]. FtsZ is a tubulin-like GTPase protein that constitutes the core of the cell division machinery in the majority of bacteria, archaea, and many eukaryotic organelles [12,162,187,264]. FtsZ-driven cell division is characterized by the initial formation of contractile rings, known as Z-rings, which are tethered to the plasma membrane by several proteins, including FtsA, ZipA and/or SepF [94,96,163,164,264]. At a later stage, both essential and dispensable proteins for cell division are recruited at the Z-rings, and the divisome is assembled. Eventually, the divisome gets activated and cell wall remodeling and peptidoglycan biosynthesis takes place [164].

Like in all prokaryotes, *S. coelicolor ftsZ* lies within a cluster of genes that together form the *division and cell wall* synthesis (*dcw*) cluster (Fig. 3). The genes include cell division genes (*fts*), genes for cell-wall synthesis (*mur*), and for septum placement and control (*sepF*, *sepG*, *divIVA*). The *ftsZ* gene itself lies in operon with two genes for proteins of unknown function, designated *ylmD* and *ylmE*, and these

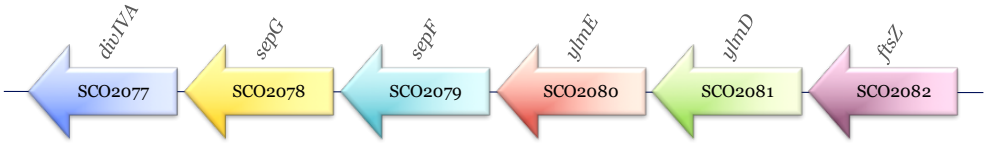

Figure 3. *Schematic representation of part of the division and cell wall synthesis (dcw) cluster of Streptomyces coelicolor A3(2).*

three genes are under the transcriptional control of the FtsZ promoter region, which consists of three promoters. It was shown that the expression of each promoter varies in response to the specific growth phase of *S. coelicolor* [78]. The gene SCO2077 encodes DivIVA, a coiled-coil protein involved bacterial cell shape determination [267]. DivIVA promotes branching and localizes at the apical sites of peptidoglycan biosynthesis of a vegetative hyphae [76,101]. YlmG (SCO2078) is a 94-aminoacid, membrane protein that interacts directly with the small SsgA (sporulation of *Streptomyces griseus*)-like protein SsgB, but not with SsgA or FtsZ, and that is required for proper septum localization, localization of DNA during sporulation [289]. Another gene in the *dcw* cluster, SCO2079, was shown to encode for SepF, a membrane tethering protein for Z rings [59]. The last two genes in the *dcw* cluster are *ylmE* (SCO2080) and *ylmD* (SCO2081), which are involved in the control of sporulation-specific cell division [290]. By sequence similarity, *ylmE* is thought to encode for a pyridoxal phosphate (PLP)-dependent alanine racemase, while *ylmD* is a putative zinc- or copper-binding laccase similar to YfiH from *Shigella flexneri* [135]. However, the exact function of YlmE and YlmD still needs to be elucidated.

Peptidoglycan biosynthesis

Peptidoglycan is an important component of the bacterial cell wall, and it has several functions, from maintenance of cell shape and turgor, to anchoring other components of the cell envelope such as proteins and teichoic acids, and protection from external threats [56,193,263]. Peptidoglycan consists of glycan strands of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) units bound to each other *via* a β(1-4) bond. Glycan strands are cross-linked by short peptides bridges that are covalently attached to the D-lactoyl group of MurNAc residues. The typical peptide bridge is L-Ala/γ-D-Glu/*meso*-A_2pm (or L-Lys)/D-Ala-D-Ala, where A_2pm stands for 2,6-aminopimelic acid [263]. The last D-Ala residue is eventually lost in the mature peptidoglycan, when a cross-link is made between the carboxyl group of the D-Ala at position 4 and the amino group of the diamino acid at position 3 [161,263]. The D-Ala-D-Ala dipeptide at the end of the penta-peptide is conserved in all prokaryotes. However, D-amino acids are not environmentally available, but must be produced by microorganism from their more abundant L-enantiomers. In bacteria, the racemization of alanine is catalyzed by

very conserved enzymes called alanine racemases (Alr's) [231]. The racemization of alanine by Alr enzymes requires one essential form of vitamin B_6, which is the pyridoxal phosphate (PLP). Genome annotation revealed the presence of two putative Alr's in *S. coelicolor* that are encoded by the genes *alr* (SCO4745) and *ylmE* (SCO2080). By sequence similarity, both *alr* and *ylmE* encode for Fold Type III, PLP-dependent proteins. A role of *ylmE* in alanine racemization would fit with the close localization of *ylmE* to *ftsZ* in the *dcw* cluster.

Vitamin B_6

Vitamins are a group of organic compounds that are nutritionally essential for animals and humans, who must absorb them through their diet. The word *vitamin* came from the contraction of the locution "vital amine" that was used by Rudolph Peters (1889-1982) and colleagues to refer to the rat "antineuritic factor" (thiamine or vitamin B_1) [92,226]. In 1932, while isolating vitamin B_1 from rice-polishings, Ohdake came across a by-product which was later identified as vitamin B_6 [200,201]. It was later found out that vitamin B_6 could cure "rat acrodynia", a condition characterized by epileptiform convulsions, microcytic anemia, and skin lesions similar to pellagra skin lesions that developed in rats fed on a semisynthetic diet with the only addition of vitamin B_1 and riboflavin [93]. Today, vitamin B_6 is a general name for a group of six interconvertible vitamers: pyridoxine (PN, generally known as vitamin B_6), pyridoxamine (PM), pyridoxal (PL), and their 5'-phopshorilated species (PNP, PMP, and PLP, respectively) (Fig. 4) [229].

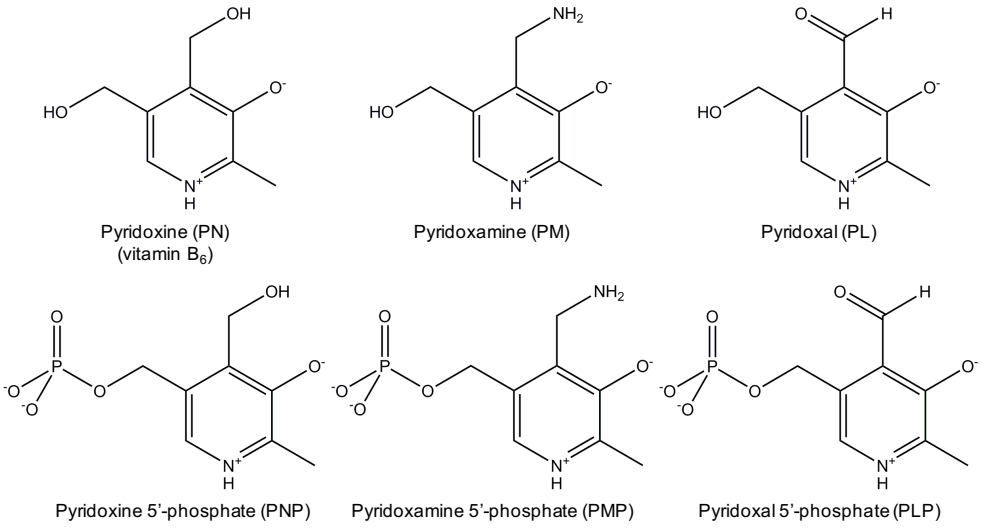

Figure 4. *Chemical structures of the vitamin B_6 vitamers.*

PLP is the most studied vitamer since it is the biologically active form of vitamin B_6 and one of the most versatile organic catalysts. PLP was shown to have antioxidant properties and could potentially have a role in both biotic and abiotic stress response as a reactive oxygen species (ROS) scavenger [14,60]. PLP and PN have also been found to have a regulatory function on membrane ion transporters [44,144], to bind steroid receptors [260] and to modulate transcription factors [202,281]. PLP can also prevent gross chromosomal rearrangements in the yeast *Saccharomyces cerevisiae*, and ensure genome stability by preventing uracil incorporation into DNA (in the absence of PLP there is accumulation of uracil in the cell) [128].

Bacteria can catalyze the *de novo* synthesis of PLP by two different pathways, that are one deoxyxylose 5'-phosphate (DXP)-dependent and one DXP-independent [181]. The DXP-dependent pathway was well characterized in *E. coli*, and it starts with the PdxA-mediated oxidation of 4-phosphohydroxyl-L-threonine (4HPT) into 3-amino-1-hydroxyacetone 1-phosphate (AHAP), and the condensation of AHAP and DXP into PNP by PdxJ. PNP is then oxidized into PLP by PdxH *via* the salvage pathway [181,248]. The DXP-independent pathway is found in bacteria, archaea, and eukarya, and it relies on PdxS and PdxT for the direct synthesis of PLP from ribose 5'-phosphate or ribulose 5'-phopshate with either glyceraldehyde 3'-phosphate or dihydroxyacetone phosphate and glutamine [181,217]. The different B_6 vitamers can also be interconverted into each other *via* the salvage pathway. In *E. coli*, the salvage pathway includes two kinases, PdxK and PdxY, that can phosphorylate PN, PM, and PL into their 5'-phosphorilated forms, and PdxH that catalyze the oxidation of PNP, and with less preference of PMP, to PLP [188,229,248].

Pyridoxal phosphate (PLP)-dependent enzymes

Pyridoxal phosphate (PLP)-dependent proteins are a group of enzymes mainly involved in the modification of amino compounds, and catalyze reactions that go from transamination, to racemization, α-decarboxylation, aldol cleavage, β- and γ-eliminations, and replacement reactions [180,229]. Although the evolutionary history of PLP-dependent enzymes can be confined to just a few lineages, their catalytic specificity diverged enough to cover more than 160 different reactions [28,211]. The high catalytic versatility of PLP is mainly thanks to its strong electrophilic behavior which makes it a sort of electron sink, able of stabilizing a great variety of carbanionic intermediates [63,251]. In all enzymes, PLP is covalently bound *via* an internal aldimine bond between the carbonyl aldehyde of PLP and the ε-amino group of a lysine. Upon substrate binding, the internal aldimine is replaced by an external aldimine (Shiff base) between the cofactor and an amino group of the substrate [63]. The specificity and the direction of the reaction totally depend on the residues surrounding the PLP cofactor, which

contribute to creating the large variety of chemical reactivity observed for the vitamin B_6. Since PLP-depending enzymes are involved in such a broad spectrum of functions, deregulation of the cellular metabolism of PLP is connected to growth inhibition, lowered stress tolerance, reduced glucan production, and biofilm formation [156]. Much research has been directed at the understanding of PLP functions and mechanism of action. However, our knowledge is still limited regarding the control of cellular homeostasis of vitamin B_6, synthesis, delivery to B_6-dependent enzymes, and degradation of PLP [227,229].

Aim and outline of the thesis

Full understanding of the inhibition mechanism of BlaC by the main β-lactamase inhibitors is an essential requirement for developing effective cures that may also prevent the onset of resistance in Mtb. Here, we set out to study BlaC inhibition by the β-lactamase inhibitors clavulanate, sulbactam, tazobactam, and avibactam. In chapter 2, the structure of wild-type BlaC is presented and compared to the structure of BlaC in complex with phosphate. Phosphate showed specific binding to conserved residues close to the active site, and may play a crucial role in triggering the recovery of β-lactamase activity after inhibition by clavulanic acid. BlaC complexes with the four β-lactamase inhibitors clavulanate, sulbactam, tazobactam, and avibactam were obtained by BlaC crystal soaking. For each inhibitor, covalent acyl-enzyme adducts were trapped and solved by X-ray crystallography, and the structures are discussed in chapter 3. Formation of BlaC covalent adducts with β-lactamase inhibitors occurs quickly upon crystal soaking, and consequently little is known about the Michaelis-Menten, pre-acylation states of BlaC and each inhibitory molecule. A possible way to fill this knowledge gap is proposed in chapter 4, which is focused on the crystal structures of BlaC mutants BlaC S70A and S70C. Both mutants carry a substitution of the active site Ser70, that renders them catalytically inactive. Soaking of BlaC S70A and S70C with β-lactamase inhibitors might provide insights into BlaC in pre-acylation complex with inhibitors. However, further optimization of crystallization and soaking conditions is required to achieve our goal.

The second part of the thesis is focused on the structural and biochemical characterization of Alr (chapter 5) and YlmE (chapter 6 and 7) from *S. coelicolor*. Alr is shown to be an alanine racemase of *S. coelicolor*. Structurally, Alr resembles homologous Alr's from other bacterial sources, including that of the D-cycloserine-resistant *Streptomyces lavendulae*. The crystal structure of YlmE (SCO2080) is presented in chapter 6. YlmE shows structural and sequence similarity to the *N*-terminal domain of Alr, but no alanine binding could be detected for YlmE. Instead, chapter 7 shows the nucleic acid binding of YlmE, which opens the way to a better understanding of the cellular role of this protein.

Data and results are summarized and discussed in chapter 8.