Current Research in Microbiology

Chapter 2

Actinobacteria from less explored ecosytems: A promising source for anti TB metabolites

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Abstract

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a highly prevalent infectious disease with almost one-third of global population believed to be infected. According to statistics, India is one among the 22 high burden countries in terms of TB incidence rates. Emergence of drug resistance among *M. tuberculosis* isolates and long term therapy using combination of drugs for its treatment are the major problems in TB control. Hence, there is an urgent need for new anti-tubercular drugs to fight against drug resistant *M. tuberculosis* strains. These drugs are expected to have least side effects and improved pharmacokinetic properties with extensive and potent activity against drug resistant strains and/or should be able to reduce the total duration of treatment. Secondary metabolites from microbial sources have a long history in the treatment of TB. Actinobacteria - the group of Gram-positive filamentous bacteria, are the promising source for secondary metabolites which produce about one third of the antibiotics available in the market. Out of 33,500 microbial bioactive metabolites reported during 1940-2010, about 13,700 are reported to have been synthesized by actinobacteria in which most of them are from terrestrial origin. In recent years, actinobacateria from rare ecosystems have been recognised as most efficient groups of secondary metabolite producers with wide range of biological activities. This book chapter describes the TB burden and anti-TB agents that can be explored from actinobacteria which serves as a promising source.

1. Tuberculosis

Tuberculosis is a contagious airborne disease caused by the pathogen *Mycobacterium tuberculosis* (Mtb) [1]. According to World Health Organization (WHO), 9.6 million people are estimated to have fallen ill with TB in which around 123,000 patients developed Multiple Drug Resistance tuberculosis (MDR-TB) and 1.5 million died from the disease in year 2015. Thus TB poses a serious problem worldwide attributing to the increase in the rate of HIV-related TB, pediatric TB, latent TB, MDR- TB and Extensively Drug Resistant TB (XDR-TB). The real challenge has been to find a new drug effective against both replicating and non-replicating *M. tuberculosis*. Hence, there is a need for new molecules to fight against TB.

1.1. General characteristics of *M. tuberculosis*

Mycobacterium tuberculosis is a slow-growing, aerobic rod-shaped acid-fast bacterium (**Figure 1**). This bacterium is a highly contagious facultative intra-cellular parasite, usually of macrophages and has a slow generation time of 15-20 hours [2]. *M. tuberculosis* strains are very weak Gram-positive. Members of mycobacterial species contain a unique lipid-rich cell wall composition that allows them to take basic dyes and resist decolourization with acid-alcohol and so are called acid fast bacilli. Acid-fast bacilli appear pink against a blue back-ground when stained by Ziehl-Neelson staining. An agar based Middlebrook medium and egg based Lowenstein-Jensen (LJ) medium are the two important solid media commonly used to grow *M. tuberculosis*. The colonies are small and buff colored when grown on either medium. It takes 2-8 weeks to get visual colonies on either type of media. The cell wall structure of *M. tuberculosis* is unique among prokaryotes and it is a major determinant of virulence for the bacterium. The cell wall has high lipid content and allows the bacteria to survive within the macrophages. It also provides the organism with a resistant barrier to many common drugs [3, 4]. The cell wall mycolic acids are considered to be a significant determinant of virulence in *M. tuberculosis*. More complete understanding of the biosynthetic pathways and gene functions will enable the development of antibiotics to prevent formation of the cell wall which are areas of great interest [5,6].

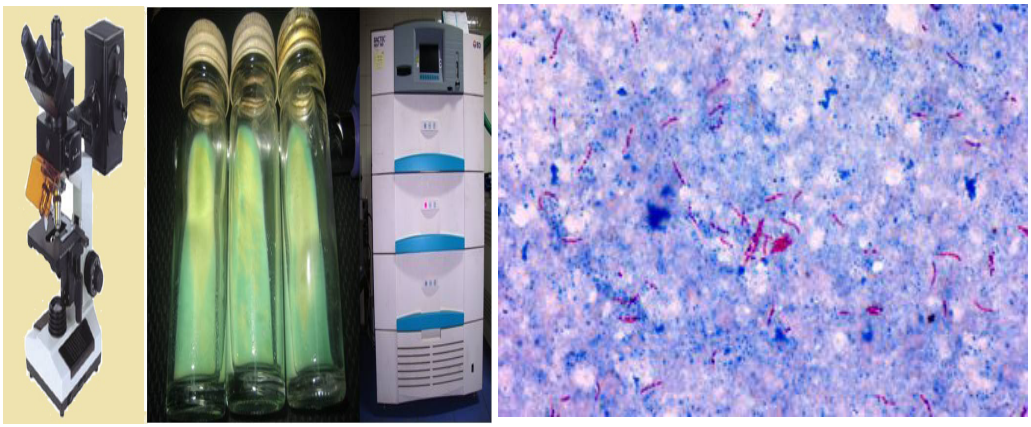


Figure 1: Cultural and micromorphology of *Mycobacterium tuberculosis*

1.2. Pathogenesis of tuberculosis

Human beings are the primary host for M. tuberculosis. Infection is spread via airborne dissemination of aerosolized bacteria containing droplet nuclei of 1-5 μm in diameter that carry *M. tuberculosis* from an individual with infectious TB disease to an uninfected individual. The infectious droplet nuclei are inhaled and get lodged in the alveoli in the distal airways. *M. tuberculosis* is then taken up by alveolar macrophages, initiating a cascade of events that result in either successful containment of the infection or progression to active disease (primary progressive TB) (**Figure 2**). Risk of development of active disease varies according to time since infection, age, and host immunity [7,8]. However, the life- time risk of disease for a newly infected young child has been estimated at 10%.

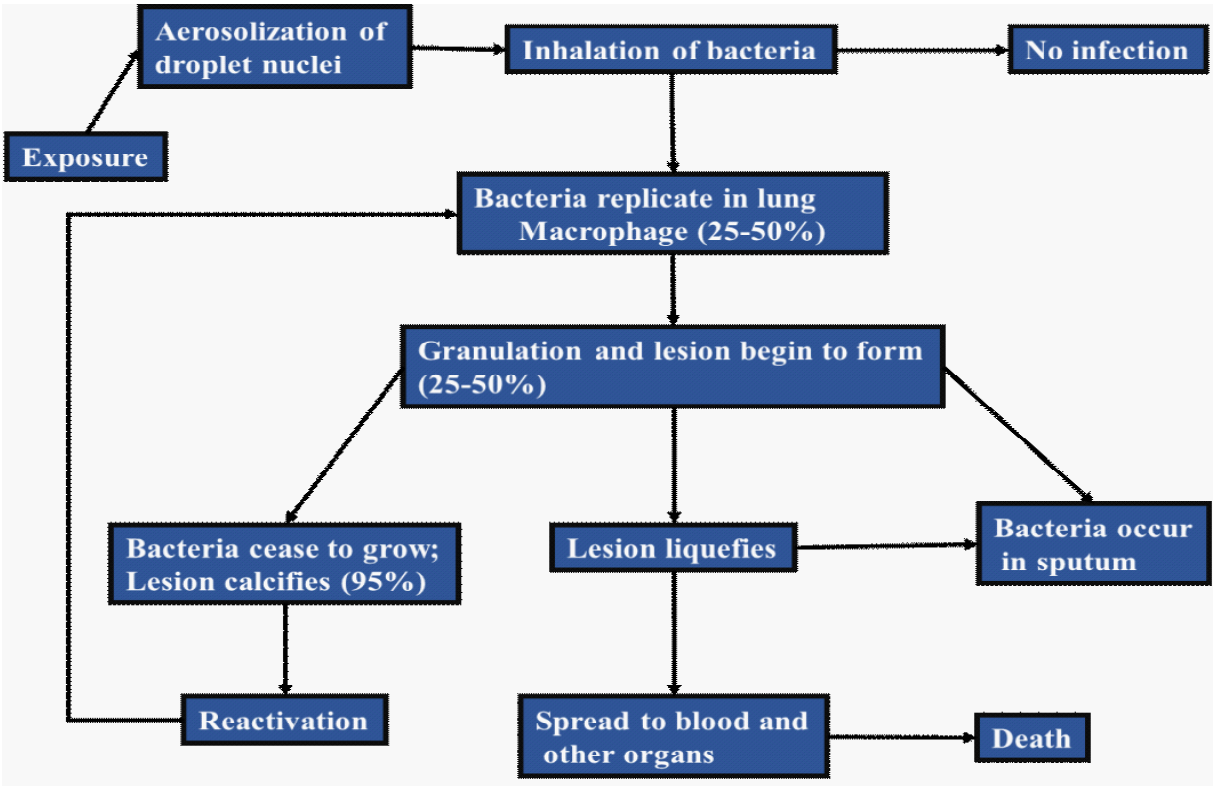


Figure 2: Pathogenesis of Tuberculosis

Latent tuberculosis infection (LTBI) is defined as a clinical condition without clinical or radiological signs of active disease and is manifested only by a positive tuberculin skin test [9]. Approximately 2 billion people or one third of the world's population, have LTBI, and approximately 10% of them will develop active TB during their life time. There is plenty of evidence that the basis for LTBI in humans is persistence of tubercle bacilli *in vivo* for long periods of time. This status is currently defined as dormancy or non-replicating persistence (NRP) [10].

1.3. Tuberculosis chemotherapy

Since the control measures for TB such as Bacillus Calmette Guérin (BCG) vaccination and chemoprophylaxis appear to be unsatisfactory, treatment by anti-TB drugs becomes

the only option available. The therapy of mycobacterial infections, in particular tuberculosis is challenging for a number of reasons. This bacterium is not susceptible to many classes of antibacterial agents. As a result, tuberculosis often requires treatment with drugs that are not commonly used for other microbial infections and often have small therapeutic windows.

The beginning of TB-chemotherapy illustrates well the importance of nature in the fight against diseases. The first drug discovered to treat TB-streptomycin (SM)-an aminoglycoside was isolated from the actinomycete, *Streptomyces griseus*, unlocking the door to the antibiotic treatment of TB [11]. While monotherapy with streptomycin was able to cure lethal forms of acute paucibacillary TB such as meningitis and miliary disease, it was soon evident that it resulted in the emergence of resistant mutants and treatment failure among patients with multibacillary forms like cavitary pulmonary TB. After the discovery of streptomycin, several synthetic drugs were introduced into the market. In 1946, Lehman from Sweden discovered para-aminosalicylic acid (PAS) as an effective TB drug. This was quickly followed in 1952 by the discovery of highly active TB drug isoniazid (INH). Both, PAS and INH ushered in the era of combination therapy. INH represented a major milestone in the tuberculosis chemotherapy because of its highly active, inexpensive nature with no significant side effect. Therapy with SM, PAS and INH prevented the selection of SM-resistant mutants and resulted in the cure of patients with 18 months of treatment. For more than 20 years this combination was the standard treatment for TB [12].

Remarkably, the nicotinamide led to the discovery of pyrazinamide (PZA) in 1952 and ethionamide (ETH)/prothionamide (PTH) in 1956. Further, screening of extracts from soil microbes led to the discovery of many other anti TB drugs viz. cycloserine, kanamycin and its derivatives such as amikacin, viomycin, capreomycin and rifamycins. Rifamycins and their derivatives are the drugs of choice for treatment of TB since 1970s [13,14].

The goals of tuberculosis treatment are to ensure cure without relapse, to prevent death, to impede transmission, and to prevent the emergence of drug resistance. Numerous antibiotics with anti-TB activity have long been classified as ‘first line’ or ‘second line’ drugs on the basis of their anti-TB activity and toxicity. First line drugs are with promising anti-TB activity and limited toxicity whereas the drugs with lesser activity and/or greater toxicity are considered as second line drugs (**Table 1**). Second-line drugs are used primarily in the treatment of patients harbouring bacilli resistant to the first-line drugs [15-18].

Table 1: Properties of first-line and second-line anti-TB drugs

Anti-TB drugs	Structure/class	Delivery route	Activity	Mechanism of action
First-line drugs				
Isoniazid	Pyridine hydrazide	Oral	Bactericidal	Inhibition of cell wall Mycolic acid synthesis and other multiple effects on DNA, lipids and NAD metabolism
Rifampin	Rifamycin	Oral	Bactericidal	Inhibition of RNA synthesis
Pyrazinamide	Nicotinamide analog	Oral	Bacteriostatic/ bactericidal	Disruption of membrane transport and energy depletion
Ethambutol	Ethylenediamine derivative	Oral	Bacteriostatic	Inhibition of cell wall arabinogalactan synthesis
Streptomycin	Aminoglycoside	IM injection	Bactericidal	Inhibits protein synthesis
Second-line drugs				
Kanamycin	Aminoglycooside	IM injection	Bactericidal	Inhibition of protein synthesis
Amikacin	Aminoglycoside	IM injection	Bactericidal	Inhibition of protein synthesis
Capreomycin	Polypeptide	IM injection	Bactericidal	Inhibition of protein synthesis
Para-aminosalicylic acid	Salicylic acid	Oral	Bacteriostatic	Inhibition of folic acid synthesis
Ethionamide	Thioamide	Oral	Bacteriostatic	Inhibition of cell wall Mycolic acid synthesis
Cycloserine	Isoxazolidinone	Oral	Bacteriostatic	Inhibition of cell wall Mycolic acid synthesis
Moxifloxacin	Fluoroquinolone	Oral or IV	Bactericidal	Inhibition of DNA replication
Gatifloxacin	Fluoroquinolone	Oral or IV	Bactericidal	Inhibition of DNA replication
Levofloxacin	Fluoroquinolone	Oral or IV	Bactericidal	Inhibition of DNA replication

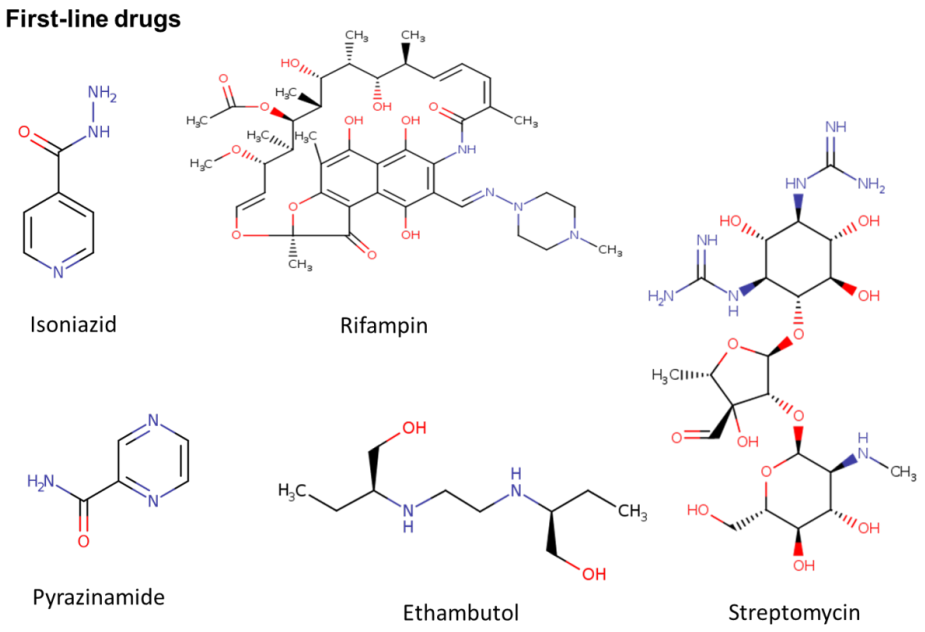


Figure 3: Structures of First line drugs used to treat drug-susceptible *M. tuberculosis*

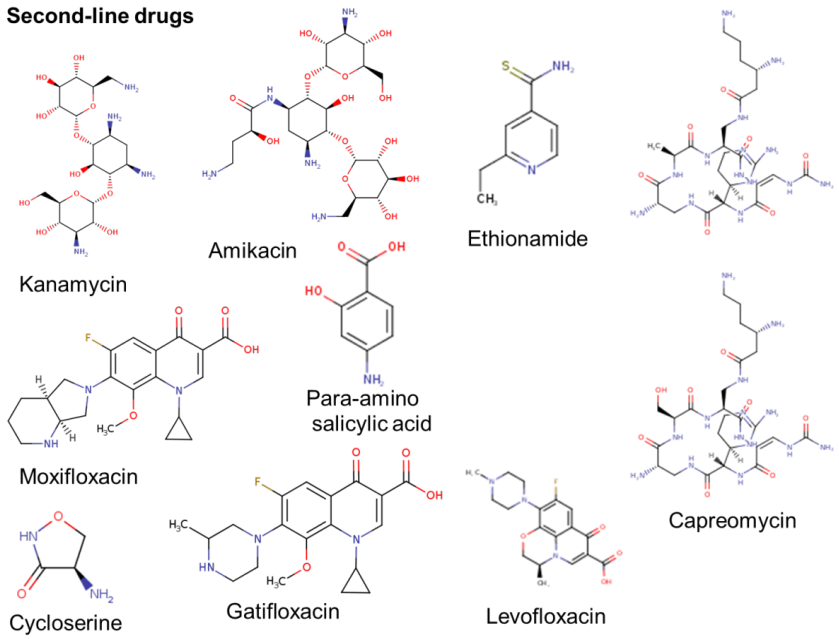
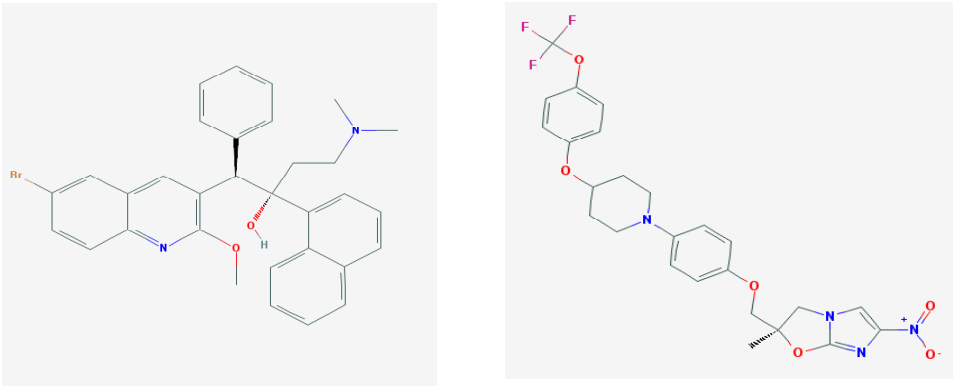


Figure 4: Structures of Second line drugs used to treat drug-susceptible *M. tuberculosis*



Bedaquiline Delamanid

Figure 5: Recently Approved Drugs for the treatment of MDR-TB infections.

As suggested by WHO, the current standard chemotherapeutic regimen for treating new pulmonary TB patients consists of a multidrug combination of the first-line drugs comprising an initial intensive phase of rifampicin (RIF), isoniazid (INH), pyrazinamide (PYZ or PZA), and ethambutol (ETB) daily for 2 months and a continuation phase of RIF and INH for a further 4 months, either daily or 3 times per week. INH and RIF are the two most potent anti-TB drugs that kill more than 99% of tubercular bacilli within 2 months of initiation of therapy [19, 20] . Using these drugs in conjunction with each other reduced the duration of anti-TB therapy from 18 months to 6 months.

Table 2: Recommended strategies for TB Therapy

	Regimen	Total duration (months)	Number of Drugs	Cost per patient
Susceptible TB	2 months INH+RIF+PZA+EMB followed by 4 months INH+RIF	6	4	USD 19-22
MDR TB Resistant pattern				USD 4000-6000 (ex works)
INH, RIF	PZA+EMB+FQN+1 SLD (entire course) + INJ (first 6 months)	18-24	5-6	
INH, RIF (EMB/PZA)	(PZA or EMB) + FQN + 2 SLD (entire course) + INJ (first 6 months)	18-24	5-6	
INH, RIF, EMB, PZA	FQN + 3 SLD (entire course) + INJ (first 6-12 months)	18-24	5-7	
INH, RIF, EMB, PZA (FQN/IN)	(INJ or FQN) + 3 SLD + 3TLD (entire course)	>24	5-7	
INH, RIF, EMB, PZA, FQN, INJ	INJ + all available SLD +TLD (entire course)	>24	5-7	

Resistance pattern is based on the results of drug susceptibility tests.
Abbreviations: INH: isoniazid; RIF: rifampicin; PZA: pyrazinamide; EMB: ethambutol; FQN: fluoroquinolones; INJ: injectable drugs (eg. Streptomycin, kanamycin); SLD: second line drugs; TLD: third line drugs (eg. Clarithromycin, amoxicillin, linezolid); All treatment administrations are performed on a daily basis

1.4. Limitations of current tuberculosis therapy

When administered appropriately, combination anti-TB therapy can be highly effective any where in the world. Regimens employing first-line drugs are orally bio-available, relatively cheap, and generally well tolerated. But this regimen is lengthy and complex, inviting non-adherence, drug interactions, drug toxicity, and treatment programs require substantial supervision to monitor adherence and tolerability [21]. New regimens for TB that could be administered for a shorter duration of therapy or more intermittently without sacrificing efficacy would reduce the burden of supervising drug administration and make treatment more widely available. Unfortunately, it is difficult to see how existing first-line drugs could be used more effectively in this regard, and there are no new agents in the later stages of the drug development pipeline [22]. In addition, the emergence of *M. tuberculosis* strains to the available drugs causes major concern which results in higher death rates, especially among HIV-infected persons.

Further, pediatric patients constitute a high risk population by *M. tuberculosis* infection. According to WHO statistics, 250,000 children develop the disease and approximately 100 000 die every year. Pharmacokinetics of several anti-TB drugs has shown poor efficacy in children. Also, there are a very limited number of anti-TB liquid formulations. Most of the first-line drugs are not available in pediatric form and they are produced only extemporaneously. For example, a liquid suspension of RIF (Rifaldin®, Sanofi-Aventis) is available in Spain. This

not only results in less compliant regimen but also makes dose-per-body weight adjustments difficult. Manipulation of solid forms (e.g. crushing and mixing with food or beverages) may lead to unpredictable changes in the stability of active compounds and their bioavailability.

1.5. Anti-TB drug resistance

The history of anti-TB drug resistance is fairly recent, emerging just over 60 years ago with the development of anti-TB drugs. For decades, the problem was identified in local areas among patients treated at reference centres in industrialized countries. With the discovery of rifampicin in 1966, and the expansion of its use between 1970 and 1990, patients who were already carriers of isoniazid resistant to *M. tuberculosis* strains became resistant to rifampicin. This was the start of a progressively growing problem which has reached epidemic proportion in some countries [23]. An individual may develop the drug resistant form of TB via inadequate therapy that enable the selection of drug resistance (acquired resistance) or infection with a drug-resistant TB strain (primary resistance).

Drug resistance in *M. tuberculosis* mostly occurs as a result of man-made selection during disease treatment. Resistance in *M. tuberculosis* develops through a limited number of mechanisms at low frequency. Mutations in the enzymes that either activate antimycobacterial drugs or are targets of drug action most commonly associated with drug resistance. Drug inactivation mechanisms that result in resistance has been of limited clinical interest because such compounds are not used in the treatment of tuberculosis. Limited number of drug efflux mechanisms has yet been described that can account for drug resistance in *M. tuberculosis*, although diffusion and transport into mycobacterial cells is an extremely important variable in drug activity. Role of these efflux pumps in clinical scenario in different patient population is not yet completely defined or explained. Episomal or transposon-mediated transfer of resistance genes into *M. tuberculosis* has not been demonstrated till date, though this is a common mechanism for the acquisition of drug resistance in other bacteria. The biochemical transformations occurring in mycobacteria during the acquisition of drug resistance are generally inferred, rather than demonstrated, and can be identified as thrust area for research in future.

The emergence of MDR-TB and XDR-TB pose serious threat to the public and further complicates the TB global emergency. They are resistant to the best antibiotics and are associated with greater morbidity and mortality than antibiotic susceptible TB. While infection with an exogenous drug resistant TB strain is related to infection control measures, the development of acquired *M. tuberculosis* is multi-faceted and can be attributed to various social, political, economic, epidemiological and pathophysiological factors [19,24]. Efforts to understand the molecular basis of *M. tuberculosis* antibiotic resistance have advanced significantly and investigations of potentially unique genetic traits in MDR- and XDR-TB strains are ongoing. Unlike other bacterial pathogens, there is no evidence that gene acquisition contributes to an-

tibiotic resistance in *M. tuberculosis*.

While MDR-TB can be effectively treated with a long-term regimen of second-line antibiotics, XDR-TB is often considered very difficult to treat, or is even untreatable, with existing chemotherapeutic agents. The diagnosis of MDR-TB or XDR-TB further subjects the patients to as many as 20 pills per day, as well as antibiotic intramuscular injections for 18-24 months. This lengthy treatment is not only more expensive than first-line antibiotics, but also comes with devastating, toxic side effects, emotional and social anxieties and psychological stresses. A large retrospective study revealed that XDR-TB cases have a worse clinical outcome than MDR-TB cases resistant to all first-line antibiotics (39% vs 54% treatment success, respectively) [19,20,25]. Therapy for LTBI is also protracted and comes in various regimens that may contain any combination of isoniazid, rifamycin, pyrazinamide and an approved fluoroquinolone, in the case of drug resistant LTBI. In order to combat MDR and XDR-TB and the overall spread of antibiotic resistant TB strains, the need for new anti-TB antibiotics is imminent. A better understanding of the mechanisms of action and development of drug resistance will allow identifying new anti-TB drug targets and better ways to detect drug resistance [26].

Table 3: Anti-TB drugs and their resistance mechanisms

Anti-TB drugs	Mechanism of action	Mechanism of resistance
Isoniazid	Inhibition of cell wall Mycolic acid synthesis and other multiple effects on DNA, lipids and NAD metabolism	KatG suppression causing decreased prodrug activation, and a mutation in the promoter region of InhA causing an overexpression of InhA
Rifampin	Inhibition of RNA synthesis	Mutation of rpoB induces a conformational change at β-subunit of RNA polymerase causing a decrease in binding affinity
Pyrazinamide	Disruption of membrane transport and energy depletion	Mutations in pncA reducing conversion to active acid form
Ethambutol	Inhibition of cell wall arabinogalactan synthesis	Mutations in embB at codon embB306
Streptomycin	Inhibits protein synthesis	Mutations in rpsL and rrs confer binding site modulation
Amikacin/ Kanamycin	Inhibition of protein synthesis	16S rRNA target site modulation (1400 and 1401 rrs gene) Increased drug inactivation via overexpression of eis aminoglycoside acetyltransferase
Capreomycin	Inhibition of protein synthesis	Cross-resistance with aminoglycosides plus mutation of tlyA which decreases rRNA methyltransferase activity
Para-aminosalicylic acid	Inhibition of folic acid synthesis	Mutations in the thyA causing a decrease in activated drug concentrations and folC mutations which cause binding site mutations

Ethionamide	Inhibition of cell wall Mycolic acid synthesis	Mutations in ethA and inhA causing decreased prodrug activation and InhA mutations which cause binding site mutations
Cycloserine	Inhibition of cell wall Mycolic acid synthesis	Overexpression of alrA decreasing drug efficiency
Moxifloxacin/ Gatifloxacin	Inhibition of DNA replication	Chromosomal mutations in the quinolone resistance-determining region of gyrA or gyrB. The most frequent mutations found are at position 90 and 94 of gyrA

1.6. Global portfolio of candidate anti-TB drugs in clinical development

After the discovery and development of new anti-TB drugs flourished in the mid-1900's, the TB drug pipeline was reduced to a mere leaky faucet with the new classes of antibiotics virtually nonexistent. It has been more than 40 years since the last novel TB-specific antibiotics were introduced into clinical practice. Most of the drugs being used to treat tuberculosis were discovered before 1950s. However, recently the US FDA approved bedaquiline for MDR-TB and delamanid as a compassionate care option for XDR-TB and TDR-TB infections. The fact is that these drugs have pronounced issues, including hERG toxicity concerns, as well as multiple ADME issues due to their high lipophilicity. It is mandated that bedaquiline has to be used only in patients who do not have other treatment options [13,16,27-29].

Given the challenge of treating MDR and XDR-TB, there are some new classes of antibiotics in the current anti-TB pipeline. There are at least 20 drugs in various stages of clinical evaluation for TB till June 2017. These can be divided into several categories: i) novel drugs being developed for TB treatment, ii) current first line TB drugs being re-evaluated to optimize their efficacy and iii) currently licensed drugs for other indications and next /generation compounds of the same chemical class being re-proposed for TB (**Table 4**).

Table 4: Drugs in clinical evaluation for tuberculosis

Pre-clinical		**Clinical**		
Early stage development	**GLP Tox.**	**Phase 1**	**Phase 2**	**Phase 3**
CPZEN-45 SATB082 Spectinamide-1810 SPR-720 (pVXc-486) TBI-166 TBI-223 TB-47	BTZ-043 GSK-070 TBA-7371 TBAJ-587	OPC-167832 PBTZ169 Q203	Delpazolid (LCB01-0371) SQ-109 Sutezolid	Bedaquiline (TMC-207) Delamanid (OPC-67683) Pretomanid (PA-824)

Table 5: Required properties of new anti-TB antibiotics

What a new antibiotic should do	Characteristic(s) required
Simplify treatment or reduce treatment duration	Strong (early) bactericidal and sterilizing activity Low pill count, fixed dose combinations
Have an acceptable toxicity profile	Allow for intermittent therapy Low incidence of treatment-limiting adverse events
Be active against MDR/XDR TB	No overlapping toxicity profile with other TB drugs No cross-resistance with first-line drugs
Be useful in HIV infected patients with TB	Minimal interactions with antiretroviral drugs No overlapping toxicity profile with antiretroviral drugs
Be active against latent TB	Activity against dormant bacilli Favourable toxicity profile

1.7. Anti-TB natural products

Although different types of anti-TB agents are available in world market, there is a growing interest in natural products for novel anti-TB drug discovery, due to non-specific side effects associated with synthetic therapeutics agents and unusual chemical diversity present in natural products. Natural products have been recognized as the source of most active ingredients of medicine. More than 80% of drug available in world market were natural products or inspired by them. Natural products derived scaffolds are therapeutic templates for the design of new therapeutic drugs using medicinal chemistry and computer-assisted design techniques. Thus, they have a remarkable impact on the treatment of TB in comparison with classical FDA-approved drugs such as rifampicin, kanamycin and cycloserine. Anti-TB compounds isolated from natural sources such as plants, microbes and marine organisms have been found with different skeleton chemical forms and conformations [30,31].

Plants have been used worldwide in traditional medicines for the treatment of various diseases and it is estimated that even today approximately 65-75% of the world's population rely on medicinal plants as the primary source of medicines. The phytochemical study of some of these plants has yielded a number of active natural products. Next to microorganisms, plants are the important source for anti-TB compounds. Several recent review and research articles have highlighted the underutilized potential of plant species as sources of antimycobacterial extracts and chemicals [32-34]. Among the plant-derived antimycobacterial compounds belonging to an exceptionally wide diversity of classes, alkaloids, terpenoids, coumarins, peptides and phenolics are more dominant. Of 17,500 higher plant species occurring in India only about 365 species have been evaluated so far for antimycobacterial activity [32,35].

The potential of marine organisms is well documented in the recent past. Yet, their utility for anti-TB drug discovery is still in its infancy. Till 2000, there are only two reports of *in-vitro* anti-TB activity from marine origin. Massetolide A and viscosin are cyclic depsipeptides isolated from cultures of *Pseudomonas* species isolated from a marine alga and tube worm, respectively. There are very few anti-TB compounds isolated from marine macro

organisms such as molluscs (kahalalides A and F), sponges (heteronemin), corals (litosterol) [36,37]. Bioactive substances from natural sources are available in extremely low quantities leading to limitations in using the reservoir of marine organisms for bioassay and therapy. To overcome these problems, few methodologies such as mariculture, bioreactors, sponge cell culture, genetic modification and most importantly chemical and semi-synthetic approach can be pursued. Certain anti-TB compounds produced by marine sponges (agelasine) and corals (litosterol) have been synthesized by chemical methods [38]. Unfortunately, none of the several hundreds of non-microbial natural products with antimycobacterial activity have moved forward in drug development.

Microorganisms that live together in the environment develop long-lasting methods to keep each other at bay. As a result, many of our most effective bactericidal agents have come from environmental organisms. Microbes are the most exploited sources for bioactive natural products including anti-TB compounds. Till date, more than 1000 antimycobacterial compounds have been reported from microbial sources among which actinomycetes are the best reported microbial source. The entire commercially available natural product based-anti-TB drug in current practice is only from actinomycete origin. There are very few reports on anti-TB compounds from other bacteria such as *Janthinobacterium* sp. Ant5-2 (J-PVP) and *Flavobacterium* sp. Ant342 (F-Y OP) isolated from land-locked freshwater lakes of Schirmacher Oasis, East Antarctica and *Bacillus subtilis* isolated from leaf of eggplant, China [39,40].

1.7.1. Actinobacteria

Actinobacteria are aerobic, gram positive filamentous bacteria with high G+C (Guanine+ Cytosine) containing DNA. Actinobacteria was first discovered by Ferdinand Cohn in 1875 and it was first named by Actinomyces (ray fungus) by Harz in 1877. Actinobacteria was first recognized by Gasperini in 1890 as potential destroyers of bacteria and fungi [41]. Actinobacteria grow well on simple laboratory media with different chemical composition but their growth is much slower than that of other bacterial groups. In solid medium, most actinobacteria form leathery, smooth surfaced, cottony colonies with varying sizes. Most actinobacteria genera form mycelial growth called substrate/vegetative or primary mycelium. In addition, from the primary mycelium, the secondary/aerial or reproductive mycelium grows on the surface of the medium which form asexual spores. The temperature ranging between 20^0C and 30^0C and p^H between 5.5 and 8.0 are conducive for the growth of most actinobacteria. Nearly one month of incubation is needed for the primary isolation of actinobacteria [42-44].

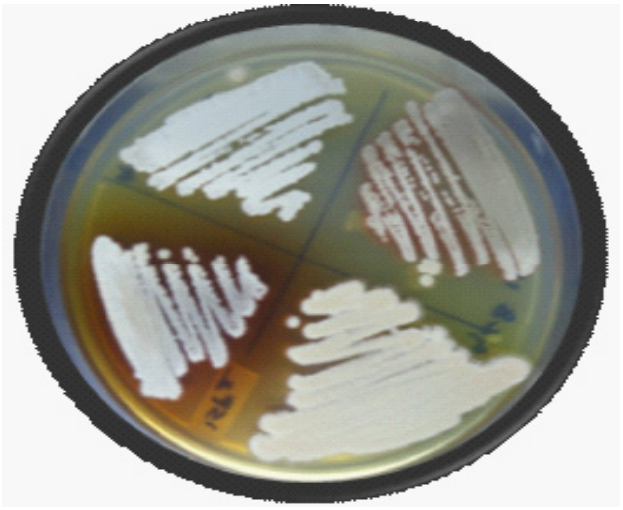


Figure 6: Cultural morphology of actinobacteria on ISP2 agar medium

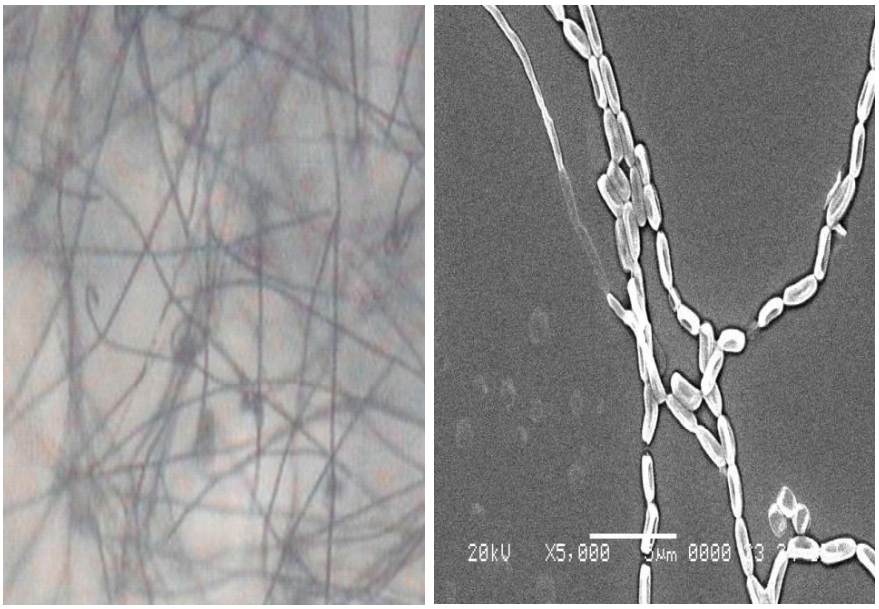


Figure 7: Micromorphology of actinobacteria (Streptomyces)

Actinobacteria are the most successful group of bacteria that occur in multiplicity of natural and man-made environments due to their ability to utilize all the available substrates in the environment. They are present in both terrestrial and aquatic ecosystems. Most of the Actinobacteria are free living saprophytes but some are parasitic or symbiotic to plants and animals. Soil is the single most reservoirs for actinobacteria. Among the total microbial population in soil, actinomycetes group occupies 10 to 50%. A single gram of rich agriculture soil can contain 10^6 colony forming units (CFU) of Streptomyces and 10^4-10^5 CFU of *Micromonospora* and other genera. The number and types of actinomycetes are highly affected by the physico-chemical properties of soil, climatic condition of that particular ecosystem, etc., [45,46].

1.7.2. Bioproducts from actinobacteria

Amongst prokaryotes, members of the order Actinomycetales, notably the genus *Streptomyces*, remain the richest source of natural products, including clinically useful antibiotics,

antimetabolites, antiparasitic, antiviral and antitumor agents [47,48]. Actinobacterial sources account for about 45% of all microbial bioactive secondary metabolites with 7600 of these compounds (80%) being produced by Streptomyces. About 74% of all actinobacteria products and 70-75% of various bacterial products exhibit antibacterial and/or antifungal activities. In contrast, only 40-45% of all fungal products have some kinds of antimicrobial activity against fungi. The antitumor activity is displayed by 30%, 24% and 27% of actinobacterial, bacterial and fungal products, respectively [47]. Despite this astonishing productivity, it has been predicted that only about 10% of the total number of natural products that can be synthesized by these organisms have been discovered [49]. The application of genomic technologies which showed that the whole genomes of *Rhodococcus* sp. RHA1, *Saccharopolysporaerythraea* NRRL 23338, *Salinisporatropica, Streptomyces avermetilis* MA-4680 and *Streptomyces coelicolor* A(3)2 contained around 20 or more natural product biosynthetic gene clusters for the production of known or predicted secondary metabolites [50-52].

The power of actinobacteria in the competitive world of chemical synthesis can be appreciated by the fact that even simple molecules are made by fermentation rather than by complex chemical synthesis. Most of the actinobacterial natural products are so complex and contain many centres of asymmetry that they will probably never be made commercially by total organic synthesis. There are five major groups of bioproducts produced by actinobacteria [53]. This includes primary metabolites, secondary metabolites, bioconversion products, microbial cell products and recombinant products (**Table 6**). Actinobacterial genera such as *Streptomyces, Rhodococcus* and *Thermomonospora* are recognized as a new source for the biosynthesis of gold and silver nanoparticles. Actinobacteria are also employed in the biodegradation of complex environmental pollutants [42,43,54,55].

Table 6: Major groups of bioproducts produced by actinobacteria

Group and Types	Some examples	Producers
Primary Metabolites		
Enzymes	Protease	*Streptomyces hygroscopicus*
	Lipase	*S. levendulae*
	Amylase	*Thermomonosporasp*
	L-asparaginase	*S. plicatus*
	L-glutaminase	*Streptomyces sp.*
Vitamins	Vitamin B_{12}	*S. olivaceus*
Amino acids	L-phenyl alanine	*Rhodococcus sp.*
	Lysine	*Nocardiaalkaloglutinosa*
Nucleotide	Guanosine	*S. griseus*
Siderophores	Madurastatin	*Actinomaduramadurae*
	Desferrioxamine B	*S. griseus*

Secondary metabolites		
Antibacterial	Streptomycin	*S. griseus*
	Chloramphenicol	*S. venezulae*
	Tetracycline	*S. rimosus*
	Erythromycin	*Saccharopolyspo-raerythraea*
	Rifampicin	*Amycolatopsismedit-teranei*
	Gentamicin	*Micromonosporaechi-nospora*
Antifungal	Amphotericin B	*S. nodosus*
	Candicidin	*S. griseus*
Antiviral	Fattiviracin	*Streptomyces sp.*
Insecticide and antiparasitic	Avermectin	*S. avermetilis*
	Milbemycin	*S. hygroscopicus*
Anticancer	Actinomycin D	*S. antibioticus*
	Mitomycin D	*S. levendulae*
Anticholestrolemic	Pravastatin	*S. carbophilus*
Growth promoter	Monensin	*S. cinnamomiensis*
	Tylosin	*S. fradiae*
Herbicide	Bialophus	*S. gradiae*
Immmuno Suppres-sive	Rapamycin	*S. hygroscopicus*
	Tacrolimus (FK506)	*Streptomyces sp*
Pigments	Actinorhodin	*Streptomyces sp.*
	Prodigiosin	*Streptoverticillium sp.*
Bioconversion products		
Antibiotic	Tetracycline	*S.aureofaciens ATCC10762*
	Streptomycin-P	*S. griseus*
Amino acid	Phenyl acetic acid	*Nocardiasp*
Polysaccharide	Chitosan	*Streptomyces sp.*
Amides	Acrylamide	*Rhodococcussp*
Microbial cell products		
Biofertilizers		*Frankiasp*
Biocontrol agents		*S. fradiae*
SCP [in animal feed]		*Thermomonosporafusca*
Recombinant products		
Antibiotics	Dihydrogranaticin	*Streptomyces sp*
	Miderrhodine A	*Streptomyces sp*

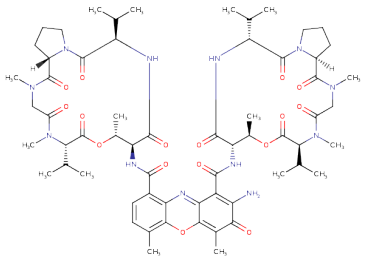
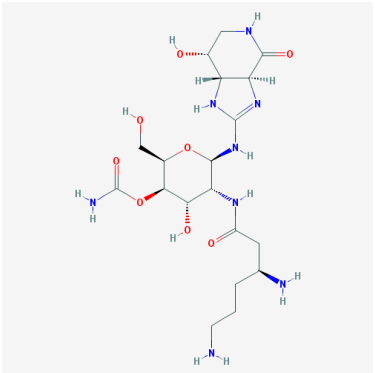
Bioactive metabolites, in particular antibiotics, production by actinobacteria is strain-specific and conditional. It has long been known that there are actinobacterial strains belonging to the same species that produce antibiotics different from one another and also that there are strains belonging to different species that produce the same antibiotic. Antibiotic production is therefore, not species specific, but strain specific [56,57].

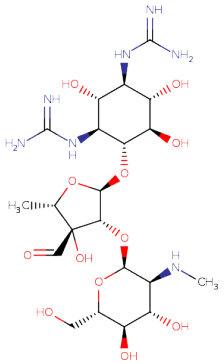
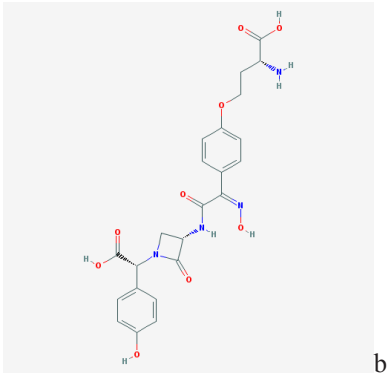
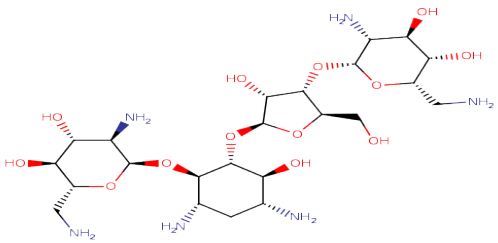
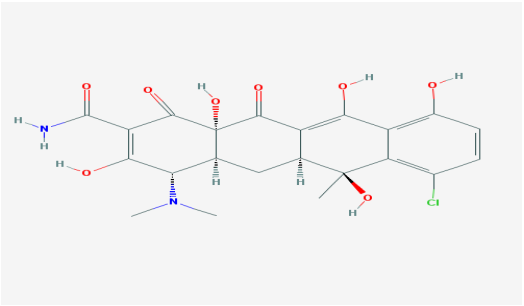
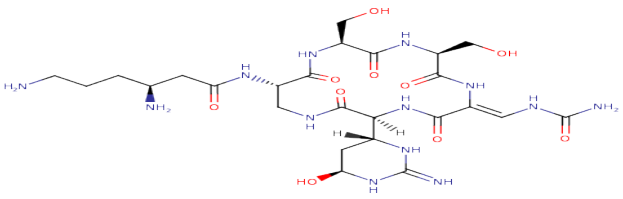
1.7.3. Anti-TB compounds from actinobacteria

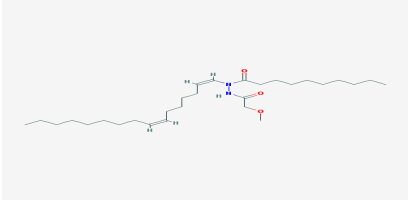
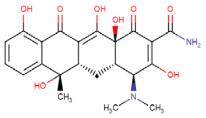
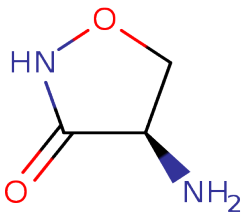
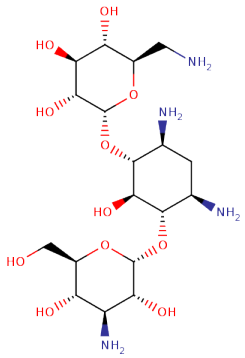
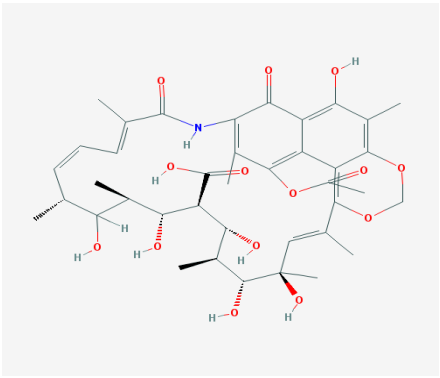
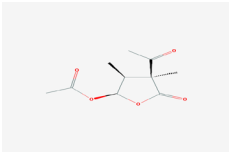
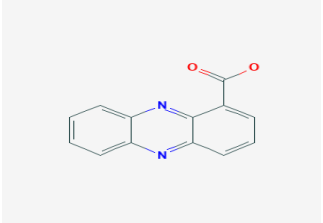
The first drug discovered to treat TB was streptomycin, an aminoglycoside isolated from the actinomycete *Streptomyces griseus*. Another outstanding example is rifampicin, an ansamycin antibiotic isolated from *Streptomyces mediterranei* renamed as *Amycolatopsismediterranei*. Following the discovery of streptomycin, in the period known as the golden era of TB research (1940-70), several synthetic drugs were introduced in the market. However, actinomycetes still played a crucial role in drug discovery against TB. For example, other aminoglycosides such as kanamycin from *Streptomyces kanamyceticus*, the semi-synthetic amikacin produced from kanamycin A and capreomycin from *Streptomyces capreolus*, as well as D-cycloserine from *Streptomyces* sp., are being used in TB treatment as second line drugs [58,59].

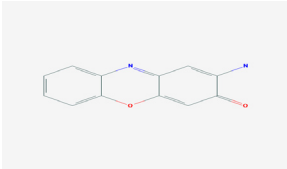
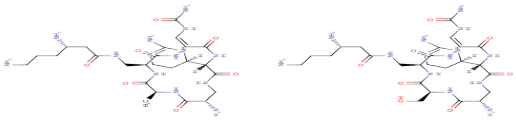
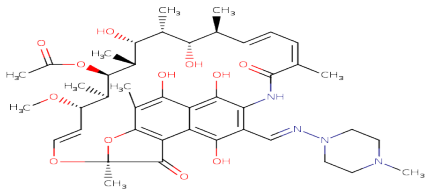
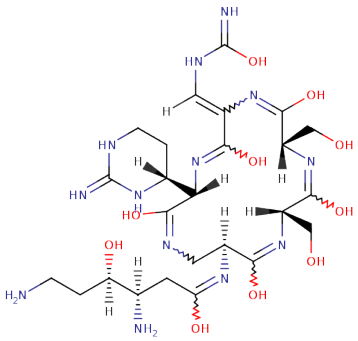
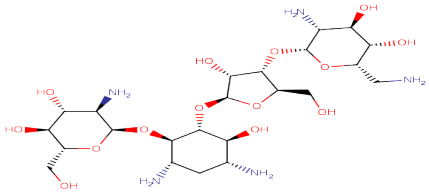
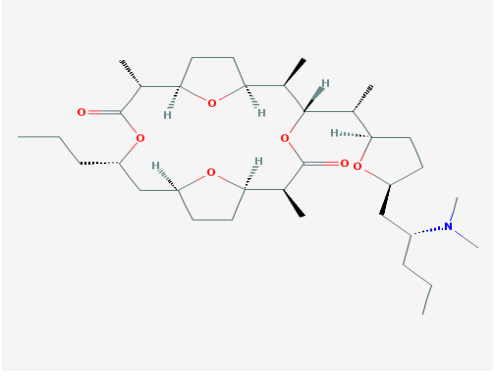
Some important anti-TB compounds isolated from actinomycetes are given in **Table 7**.

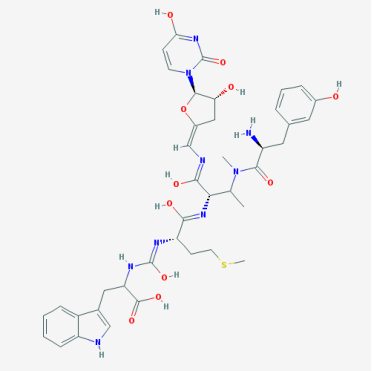
Table 7: Anti-TB antibiotics reported from actinomycetes

Anti-TB antibiotics	Chemical Structure	Producing actinomycetes
Actinomycin		*Actinomyces sp.*
Streptothricin		*Streptomyces sp*

Streptomycin		*Streptomyces griseus*
Nocardicin	 b	*Noardiacoeliaca*
Neomycin		*Streptomyces fradiae*
Aureomycin		*Streptomyces aureofaciens*
Viomycin		*Streptomyces puniceus*

Elaiomycin		*Streptomyces gelaticus*
Tetracycline		*Streptomyces rimosus*
Cycloserine		*Streptomyces orchidaceus*
Kanamycin		*Streptomyces kanamyceticus*
Streptovaricin		*Streptomyces spectabilis*
Acetomycin		*Streptomyces ramulosus*
Tubermycin		*Streptomyces misakiensis*

Questiomycin		*Streptomyces sp*
Capreomycin		*Streptomyces capreolus*
Rifamycin		*Amycolatopsismediterranei*
Tuberactinomycin		*Streptomyces griseoverticillatus*
Paramomycin		*Streptomyces sp*
Pamamycins		*Streptomyces alboniger*

Sansanmycin		*Streptomyces sp. SS*

1.7.4. Actinobacteria from rare/less explored ecosystems

Nowadays, it is becoming increasingly difficult to find novel metabolites from common actinomycetes as regular screening leads to the rediscovery of mostly known compounds. However, it is not the end of an era but an endless frontier. Using standard procedures for the isolation of novel actinomycetes from poorly studied habitats is an alternative [49] and by applying new methods, rare or uncommon actinomycetes can be isolated. Novel species may contain unique compounds as the evolution of secondary metabolites [60].

Actinobacteria are traditionally considered as organisms that cannot occupy natural ecological niches that are characterized by extreme conditions. Actinobacteria have specific environmental needs differing from those of other mycelial bacteria. Now-a-days, great amount of data on the isolation of actinobacteria resistant to extreme environmental factors like acidity, salinity, temperature and pressure has been accumulated. Actinobacteria are isolated from various rare ecosystems such as forests, mountains, deep sea, desert and alkaline soils all over the world [61-63] (**Table 8**). But there are very few reports on antitubercular compounds from actinobacteria of rare ecosystems.

Table 8: List of some novel actinobacteria isolated from rare ecosystems

Organisms	Source	Special features
Beutenbergia cavernae gen. nov. sp. nov.	Cave soil, China	-
Nocardiopsiskunsanensissp.nov	Saltern, Kunsan Republic of Korea	Halophilic
Actinopolymorphasingaporensis gen. nov. sp. nov	Tropical forest soil, Singapore	Halophilic
Nocardiopsishalotolerans sp. nov	Salt marsh soil, Kuwait	Halophilic
Nocardiopsismetallicus sp. nov	Alkaline slag dump, Germany	Alkaliphilic
Nocardiopsisalkaliphila sp. nov.	Soil from Estern Desert, Egypt	Alkalophilic
Nocardiopsisaegyptia sp. nov	Marine sediments, Abu Qir Bay, Egypt	Halophilic
Salinispora arenicola gen. nov. sp. nov.	Marine sediment	Obligate marine actinomycete
Nocardiopsisarabia sp. nov.	Sand Dune soil, Egypt	Halotolerant
Nocardiopsisvalliformis sp. nov	Alkali lake soil, China	Alkaliphilic

Gracillibacillushalophilus sp. nov	Saline soil, Qaidam Basin, China	Halophilic
Saccharopolysporaqijiaojingensis sp. Nov	Salt lake, North west China	Halophilic
Amycolatopsis marina sp. nov	Ocean sediment of South China Sea	Halophilic
Nocardiopsis terrae sp. nov.	Saline soil, China	Halophilic
Streptomyces sannunensis sp. nov.	WadiSannur, Egypt	Alkalophilic
Yahishiella deserti gen. nov. sp. nov.	Desert soil,	
China	Thermotolerant	
Verrucosisporawenchangensis sp. nov.,	Mangrove soil, China	-
Thermoactinospora rubra gen. nov. sp. nov.,	Tengchong National volcanic geological park, China	Thermophilic

1.7.5. Anti TB activity of actinobacteria isolated from rare/less explored sources

More compounds from actinobacteria of terrestrial and marine origin are still in different stages of investigation to be developed as potential anti-TB drugs. Some of the reports on anti TB activity of actinobacteria from rare / less explored ecosystems are described below.

Cyclomarin A, a novel anti-inflammatory cyclic peptide, is one of three cyclomarin compounds isolated from marine *Streptomyces* spp. CNB-982. Recently, cyclomarin A was revived as an anti-TB lead compound based on results of whole cell-based screening assays [64]. Cyclomarin A is composed of seven amino acids, of which two are common amino acids (alanine and valine), and five are unusual amino acids [N-methylleucine, N-methylhydroxyleucine, β-methoxyphenylalanine, 2-amino-3,5-dimethylhex-4-enoic acid, and N-(1,1-dimethy l-2,3-epoxypropyl) β-hydroxytryptophan] [65].

In another study, crude bioactive compounds from 15 actinobactreial strains isolated from rare marine and forest ecosystems was produced by shake flask fermentation using soybean meal medium. Culture supernatant and mycelia were extracted with ethyl acetate and methanol, respectively. Antibacterial activity of crude extracts was tested by disc diffusion method against gram positive and gram negative bacteria. Actinobacterial strains D10, D5, NEK5, ANS2, M104 and R2 showed prominent activity. Culture filtrates and crude extracts were tested against standard strain Mycobacterium tuberculosis H37Rv and drug sensitive and drug resistant clinical isolates of *M. tuberculosis* by luciferase reporter phage (LRP) assay. Considerable variation was observed in antimycobacterial activity between actinobacterial culture filtrates and solvent extracts. Actinobacterial strains viz., D10, D5 (desert), CSA14 (forest), CA33 (alkaline soil), NEK5 (Neem plant), MSU, ANS2, R2 and M104 (marine) screened in the present study were found to be highly potent showing good antibacterial and antimycobacterial activity. Five of them such as A3, CSA1, EE9, ANS5 and R9 were exclusively active against *M. tuberculosis*. Secretary products of actinobacteria of rare ecosystems are meant to antagonize organisms in their respective environments. These are likely to be novel antimycobacterial compounds as they unknown to human pathogens [66].

Crude bioactive comounds produced from 14 facultative psychrophilic actinobacteria were screened for antimycobacterial activity against *Mycobacterium tuberculosis*. The results showed that, four strains were active against the test organism *M. tuberculosis*. The active isolates screened in the present study were found to be highly effective and comes under Streptomycetes species (RH7 and RH8), *Micromonospora* species (RH9) and *Micropolyspora* species (RH12). All the isolates capable of producing metabolites and their presence were confirmed by TLC. The 'active culture filtrate' showed 'only one band' and its functional group is low molecular weight neutral compounds and amines were determined based on their solubility and pH [67].

Bioactive potential of actinobacteria isolated from certain less explored Indian ecosystems was tested against *Mycobacterium tuberculosis* and other non-mycobacterial pathogens. Actinobacteria were isolated from the soil samples collected from desert, coffee plantation, rubber forest, and hill area from Western Ghats and Eastern Ghats Ecosystems in India and their cultural and micro morphological characteristics were studied. Crude extracts were prepared by agar surface fermentation and tested against *M. tuberculosis* isolates by luciferase reporter phage (LRP) assay at 100 μg/mL. Activity against non-mycobacterial pathogens was studied by agar plug method. Totally 54 purified cultures of actinobacteria including 43 *Streptomyces* and 11 non *Streptomyces* were isolated. While screening for antitubercular activity, extracts of 39 actinobacteria showed activity against one or more *M. tuberculosis* isolates whereas 27 isolates exhibited antagonistic activity against non mycobacterial pathogens. In particular crude extracts from sixteen actinobacterial isolates inhibited all the three *M. tuberculosis* isolates tested. Findings of the present study concluded that less explored ecosystems investigated in this study are the potential resource for bioactive actinobacteria. Further purification and characterization of active molecule from the potential extracts will pave the way for determination of MIC, toxicity, and specificity studies [68].

Five new nucleoside antibiotics, named streptcytosines A–E, and six known compounds, de-amosaminyl-cytosamine, plicacetin, bamicetin, amicetin, collismycin B, and SF2738 C was isolated from a culture broth of *Streptomyces* sp. TPU1236A collected in Okinawa, Japan. The structures of new compounds were elucidated on the basis of their spectroscopic data (HRFABMS, IR, UV, and 2D NMR experiments including ^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY spectra). Streptcytosine A belonged to the amicetin group antibiotics, and streptcytosines B–E were derivatives of de-amosaminyl-cytosamine, 2,3,6-trideoxyglucopyranosyl cytosine. Streptcytosines A inhibited the growth of *Mycobacterium smegmatis* (MIC=32 μg/mL), while compounds streptcytosines B-E were not active at 50 μg/disc. Bamicetin and Amicetin showed the MICs of 16 and 8 μg/mL, respectively [69].

The feasibility and relevance of screening a library of raw actinomycete extracts (ECUM library) for the identification of antituberculosis activities was assessed on 11,088 extracts us-

ing a multiple-screening approach. Each extract was first tested at two concentrations against non-infected macrophages as a control, then against *Mycobacterium tuberculosis* growing in broth medium as well as infecting murine macrophages. The screening results indicated a library of good quality with an apparent low proportion of cytotoxic extracts. A correlation was found between both bacterial assays, but the intracellular assay showed limitations due to low rates of cell survival. Several extracts of interest were highlighted by this multiple screening. A focus on the strain producing the two most effective revealed similarities with known producers of active molecules, suggesting the possibility of selecting relevant extracts using this strategy.

Microbes belonging to the genus *Verrucosispora* possess significant chemical diversity and biological properties. They have attracted the interests of many researchers and are becoming promising resources in the marine natural product research field. A bioassay-guided isolation from the crude extract of *Verrucosispora* sp. strain MS100047, isolated from sediments collected from the South China Sea, has led to the identification of a new salicylic derivative, glycerol 1-hydroxy-2,5-dimethyl benzoate , along with three known compounds, Brevianamide F, Abyssomicin B, and Proximicin B. Compound 1 showed selective activity against methicillin-resistant *Staphylococcus aureus* (MRSA) with a minimum inhibitory concentration (MIC) value of 12.5 μg/mL. Brevianamide F, which was isolated from actinobacteria for the first time, showed a good anti-BCG activity with a MIC value of 12.5 μg/mL that has not been reported previously in literatures. Proximicin B showed significant anti-MRSA (MIC = 3.125 μg/mL), anti-BCG (MIC = 6.25 μg/mL), and anti-tuberculosis (TB) (MIC = 25 μg/mL) activities. This is the first report on the anti-tubercular activities of proximicins.

In addition, *Verrucosispora* sp. strain MS100047 was found to harbor 18 putative secondary metabolite gene clusters based on genomic sequence analysis. These include the biosynthetic loci encoding polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) consistent with abyssomicins and proximicins, respectively. The biosynthetic pathways of these isolated compounds have been proposed. These results indicate that MS100047 possesses a great potential as a source of active secondary metabolites [70].

Cyclomarin A, a novel anti-inflammatory cyclic peptide, is one of three cyclomarin compounds isolated from marine *Streptomyces* spp. CNB-982. Recently, cyclomarin A was revived as an anti-TB lead compound based on results of whole cell-based screening assays. Cyclomarin A shows antibacterial activity against replicating M. tb in culture broth and in human-derived macrophages, with minimal inhibitory concentration (MIC) values of 0.3 and 2.5 μM, respectively. Exposure to 2.5 μM cyclomarin A for 5 days resulted in 90 % kill in non-replicating M. tuberculosis. Cyclomarin A is effective against MDR *M. tuberculosis*, contrary to the resistance shown by *Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus*. This activity of cyclomarin A suggests

that its cytotoxicity against *M. tuberculosis* is mediated by a novel mode of action, which distinguishes it from the existing anti-TB drugs. To identify target molecules of cyclomarin A, *M. tuberculosis* lysates was incubated with sepharose beads coupled to cyclomarin A and screened the bound proteins, which resulted in the identification of ClpC1 by proteomic analysis. In the mixture containing cyclomarin A and *M. tuberculosis* lysates, cyclomarin A was specifically bound to ClpC1 and this was confirmed by competition assays [71].

Lassomycin, an anti TB lead molecule was isolated from the rare actinobacteria Lentzeakentuckyensis spp. IO0009804. Lassomycin, refasan anti-TB lead compound, shows anti-TB activity against MDR and XDR M. tb, as well as to the drug-sensitive strain. The MIC ranges from 0.41 to 1.65 μM, and it is effective in killing inactive *M. tuberculosis* as well as exponential M. tb, unlike rifampicin, which is ineffective against inactive *M. tuberculosis* [72].

Ecumicin is a macrocyclic peptide produced by another rare actinobacteria, *Nonomuraea* spp. MJM5123. It is composed of 13 amino acids, including natural and highly methoxylated unnatural amino acids. Ecumicin shows promising anti-TB activity against MDR and XDR M. tb as well as the sensitive M. tb strain, with MIC values ranging from 0.16 to 0.62 μM. It effectively kills inactive M. tb with a minimal bactericidal concentration of 1.5 μM, indicating the possibility of shortening the duration of treatment [73].

Chrysomycin A from an actinomycete isolated from a coastal area in Kerala was purified by bioassay guided fractionation against *M. tuberculosis*. The authors reported that, for the first time, the chromomycin A has antimycobacterial activity. It was found to be bactericidal to planktonic and intracellular *M. tuberculosis* with an MIC of 3.125 μg/ml; it is non-hemolytic and has negligible cytotoxicity. The actinomycete that produces chrysomycin A was found to be a *Streptomyces* sp. through 16S rRNA gene sequencing [74].

2. Conclusion

The huge burden of tuberculosis and its associated plethora of challenges, makes it a herculean task to discover novel drugs against it. The knowledge of the problems of resistance and latency along with HIV co-infection leads to path of less explored avenues to be experimented to discover new metabolites with novel mechanism of action. Actinobacteria comes as a trusted savior for rescue as the success rate of tapping this goldmine has proved and evidenced as potential to alter the TB regimen.Thus experimental validation of the *invitro* results in animal models will pave way for preclinical studies to hasten the TB drug discovery facilitating the arrival of new drugs sooner.

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