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Review

Advances in the development of molecular genetic tools for *Mycobacterium tuberculosis*

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ABSTRACT

Mycobacterium tuberculosis, a Gram-positive bacterium of great clinical relevance, is a lethal pathogen owing to its complex physiological characteristics and development of drug resistance. Several molecular genetic tools have been developed in the past few decades to study this microorganism. These tools have been instrumental in understanding how *M. tuberculosis* became a successful pathogen. Advanced molecular genetic tools have played a significant role in exploring the complex pathways involved in *M. tuberculosis* pathogenesis. Here, we review various molecular genetic tools used in the study of *M. tuberculosis*. Further, we discuss the applications of clustered regularly interspaced short palindromic repeat interference (CRISPRi), a novel technology recently applied in *M. tuberculosis* research to study target gene functions. Finally, prospective outcomes of the applications of molecular techniques in the field of *M. tuberculosis* genetic research are also discussed.

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1. Introduction

Mycobacterium tuberculosis belongs to the genus mycobacterium, which comprises filamentous Gram-positive bacteria that are characterized by their complex surface-lipid compositions. In humans, *M. tuberculosis, Mycobacterium leprae*, and *Mycobacterium ulcerans* cause serious diseases including tuberculosis (TB), leprosy, and Buruli ulcer, respectively. Genus mycobacterium receives special attention because of its widespread clinical impact (Wayne and Kubica, 1986). *M. tuberculosis* is the most important pathogen among all mycobacterium species because it causes TB, one of the deadliest communicable diseases globally. According to recent WHO report on TB worldwide in 2016, 10.4 million people were infected by *M. tuberculosis* and 1.7 million deaths were caused by TB, which included patients co-infected with HIV (WHO, 2017). Treatment of TB currently is a major challenge because of the occurrence and spread of multiple drug-resistant (MDR), extensive drug-resistant (XDR), and totally drug-resistant (TDR) strains of *M. tuberculosis*. These drug-resistant strains resist the most effective TB chemotherapies that are presently available (Islam et al., 2017), and the mortality rate increases when patients are co-infected with HIV (Gandhi et al., 2006). The spread of drug-resistant strains of *M. tuberculosis* poses a major challenge for global TB management. Genetic analysis of *M. tuberculosis* is essential for understanding its complex biology, the genetic basis of therapeutic targets and mechanisms underlying drug resistance, which will facilitate the diagnosis of drug-resistant *M. tuberculosis* and the development of new anti-TB drugs and treatment regimens (Hameed et al., 2018). Genetic modifications of *M. tuberculosis* are primarily limited by its slow growth and rigid cell wall in addition to the need for expensive biosafety level 3 laboratories and a lack of efficient genetic engineering techniques as opposed to those used for other “model” organisms. Nevertheless, several methods for genetic analysis of *M. tuberculosis* have been developed over the last decades (Fig. 1). Introduction of exogenous DNA into mycobacterium was first achieved by using a shuttle plasmid (Jacobs

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et al., 1987). Integrative plasmids were developed for the integration of reporter genes and antigen genes into the mycobacterial genome (Snapper et al., 1988; Lee et al., 1991). Subsequently, genetic tools developed for mycobacteria transformation led to the establishment of versatile genetic systems (Hatfull, 1993). A thermosensitive plasmid, combining the mycobacterial thermosensitive origin of replication and *sacB* (ts-*sacB*) was well adapted for target gene knockout in *M. tuberculosis* (Pelicic et al., 1997). Generation of gene mutations in *M. tuberculosis* was made simpler by this method; however, the occurrence of spontaneous mutations in *sacB* gene limited its applications. To address this shortcoming, phage-based method to deliver the substrate DNA more efficiently was developed, and this method enabled target-gene deletion or replacement by allelic exchange in mycobacteria with higher success rates (Bardarov et al., 2002). However, this method is still not very impressive because it involves complicated procedures and is time-consuming. The development of recombineering method increased the probability of homologous recombination between an allelic exchange substrate (AES) and a target gene sequence in *M. tuberculosis* (Van Kessel and Hatfull, 2007). Recombineering can also be used to induce point mutations in the mycobacterial genome, using single-stranded DNA as a substrate (Van Kessel and Hatfull, 2008). Furthermore, combination of phage-based AES delivery and recombineering systems yielded higher success rates for gene knockout in *M. tuberculosis* (Tufariello et al., 2014; Jain et al., 2014). Mycobacteriophages have become a popular method for target gene deletion in mycobacteria and have been used for diagnosis of TB, anti-TB therapy, and determination of drug susceptibility. Transposon mutagenesis is yet another efficient approach to identify virulence factors and genes essential for growth (Beste et al., 2009; Barczak et al., 2017). This approach was improved by using unique DNA sequence tags attached to transposons, facilitating the identification of individual mutant clones. Hence, numerous mutants were screened *in vivo* using only a few experimental animals (Camacho et al., 1999). Transposon mutagenesis combined with high-throughput sequencing has been used for genome-wide identification of essential genes in *M. tuberculosis* (Griffin et al., 2011; Zhang et al., 2012). The development of genome editing approaches has facilitated the understanding of mechanisms underlying drug-resistance, the role of genes associated with survival and virulence, and the identification of therapeutic targets in *M. tuberculosis* (Casali et al., 2014; Mendum et al., 2015; Quigley et al., 2017). In recent years, the clustered regularly interspaced short palindromic repeat interference (CRISPRi) system has been used for investigating the functions of essential genes in *M. tuberculosis* (Choudhary et al., 2015; Singh et al., 2016; Rock et al., 2017). A remarkable achievement was the sequencing of the *M. tuberculosis* genome which led to a better understanding of the complex biology of this pathogen. Next-generation sequencing (NGS) technology has been widely used for studying the molecular epidemiology of TB; however, the quality of *de novo* assemblies of small microbial genomes is not optimal and analysis of larger structural variations is difficult. Third-generation sequencing technologies overcame these limitations and have markedly increased our understanding of the mycobacterial genomes. Concurrently, it also facilitated genome-wide detection of methylated bases in mycobacteria (Zhu et al., 2016). This review discusses the advancements in the development of new molecular genetic

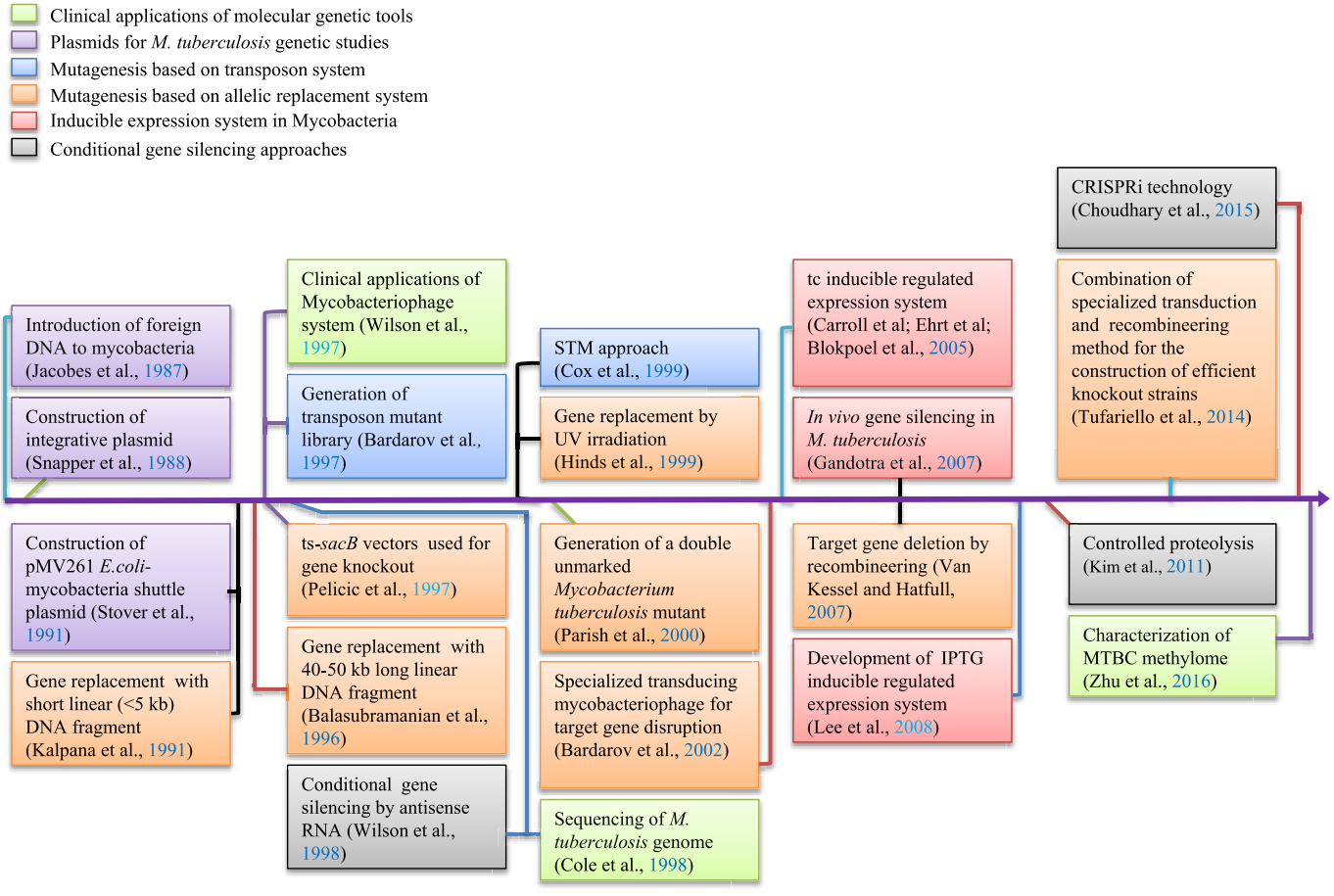

Fig. 1. Chronological advancements in the development of molecular genetic tools to study *M. tuberculosis*.

techniques and their future applications in *M. tuberculosis* genetic research.

2. Plasmid vectors for *M. tuberculosis* genetic studies

Many bacteria carry their own natural plasmids. However, in the case of mycobacteria, only *Mycobacterium scrofulaceum*, *Mycobacterium fortuitum, Mycobacterium avium*, *Mycobacterium ulcerans* and *Mycobacterium abscessus* harbor plasmids and no natural plasmids have been reported in *M. tuberculosis* to date (Labidi et al., 1985; Jucker and Falkinham, 1990; Zainuddin and Dale, 1990; Stinear et al., 2004; Leão et al., 2013). In mycobacteria, the construction of plasmids and/or cloning vectors has three basic requirements: (i) the ability to replicate in *E. coli* and mycobacteria or to integrate into mycobacterial genome; (ii) the presence of multiple cloning sites containing suitable restriction enzyme sites; and (iii) the presence of a suitable selectable marker. Based on their ability to replicate in both *E. coli* and mycobacteria, most of these plasmids are shuttle plasmids that are broadly categorized in two families: pMSC262 and pAL500. pMSC262 was isolated from *M. scrofulaceum* and pAL500 from *M. fortuitum* (Labidi et al., 1985). Numerous extra-chromosomally replicating plasmids have been developed for *M. tuberculosis*, such as the widely used *E. coli*-mycobacterial pYUB12 and pMV261 shuttle plasmids (Snapper et al., 1990; Stover et al., 1991). In addition to extra-chromosomal plasmids, integrative plasmids are also widely used in mycobacterial research. These types of plasmids contain the integrase gene (*int*) and the phage attachment site (*attP*) from mycobacteriophages (Snapper et al., 1988). Integrative plasmids have the following advantages: (i) the integration of a foreign gene is highly efficient; (ii) the inserted gene remains in a single copy; and (iii) inheritance stability of the gene is greater in the absence of antibiotic selection. However, their integration site may affect the expression of the integrated gene because of the presence of native promoters (Murry et al., 2005). To overcome this limitation, Springer et al. (2001a) developed dual integration vectors that separately encode *attP* and *int* site. Based on this concept, other integrative vectors were developed, wherein the integration system was derived from Ms6 mycobacteriophage and *Streptomyces* ΦC31 phage (Murry et al., 2005; Dos Vultos et al., 2006). Pham et al. (2007) constructed new set of mycobacterial integrative plasmids using *attP-int* cassette derived from Tweety mycobacteriophage. These integrative plasmids are compactable with L5 integration-proficient derived plasmids, which facilities simultaneously integration of multiple genes at different chromosomal locations. Similarly, Morris et al. (2008) developed integration-proficient plasmids containing *attP* and *int* of mycobacteriophage Giles. Saviola and Bishai (2004) reported the integration of multiple plasmids into the mycobacterial chromosome by inserting the bacterial attachment site, *attB*, into the integrating plasmid. Pashley and Parish (2003) reported a technique, to excise a resident plasmid and facilitate the integration of an incoming plasmid. However, Lewis and Hatfull (2000) identified an excisionase from mycobacteriophage L5 that facilitates recombination and is useful for chromosomal excision of integrative vectors, which can then be reused. Parikh et al. (2013) developed a series of plasmid vectors for gene expression, gene replacement, and protein-protein interaction studies in *M. tuberculosis*. Integrative plasmids have been crucial for the integration of multiple recombinant antigen genes, insertion of reporter genes into the mycobacterial chromosome, gene functional studies, and construction of mycobacterial complement strains. Other plasmids widely used for mutagenesis and gene function studies in mycobacteria are listed in Table 1.

Table 1
Common plasmids for *M. tuberculosis* genetic study.

Plasmid	Origin	Marker	Reference
pYUB12	pAL5000	kan^R	Snapper et al., 1990
pMY10	pAL5000	kan^R	Lazraq et al., 1991
pMH94	L5	kan^R	Lee et al., 1991
pMV261	pAL5000	kan^R	Stover et al., 1991
pMV361	L5	kan^R	Stover et al., 1991
pMD31	pAL5000	kan^R	Donnelly-Wu et al., 1993
pl6R1	pAL5000	hyg^R	Garbe et al., 1994
pNBV1	pAL5000	hyg^R	Howard et al., 1995
pYUB854	pAL5000	hyg^R	Bardarov et al., 1997
pYUB870	pAL5000	kan^R	Bardarov et al., 1997
pBP10	pMF1	kan^R	Bachrach et al., 2000
pYUB415	L5	hyg^R, kan^R	Pethe et al., 2001
pYUB412	L5	hyg^R	Hsu et al., 2003
pAPA3	L5	gen^R	Parish et al., 2007
pTTP1A	Tweety	kan^R	Pham et al., 2007
pTTP1B	Tweety	kan^R	Pham et al., 2007
pJV53	pAL5000	kan^R	Van Kessel and Hatfull, 2007
pGH1000A	Giles	hyg^R	Morris et al., 2008
pGH1000B	Giles	hyg^R	Morris et al., 2008
pJV62	pAL5000	kan^R	Van Kessel and Hatfull, 2008
pML1342	L5	hyg^R	Huff et al., 2010
pJV53Ts	pAL5000*	kan^R	Yang et al., 2014
pTY95	L5	hyg^R	Yang et al., 2014
p60luxN	pAL5000	hyg^R	Liu et al., 2015
p60GTE	pAL5000	gen^R, tsr^R, hyg^R	Mugweru et al., 2017
p60GTI	L5	gen^R, tsr^R,	Mugweru et al., 2017

hyg^R: hygromycin resistance; kan^R: kanamycin resistance; gen^R: gentamicin resistance; tsr^R: thiostrepton resistance. *Thermosensitive origin.

Plasmid vectors play a vital role in studies on mechanisms underlying drug resistance and in the generation of recombinant vaccines (Aldovini and Young, 1991; Stover et al., 1991; Zhang et al., 1992). An appropriate selection marker is required for the selection of mycobacterial cells that carry the recombinant DNA. Importantly, the transformation efficiency of mycobacterial species is different, depending on the plasmid and the marker gene used (Garbe et al., 1994). Unfortunately, *M. tuberculosis* is naturally resistant to many antibiotics which limit the marker choices (Parish and Brown, 2008). Snapper et al. (1988) first used the aminoglycoside phosphotransferase gene, which confers resistance to kanamycin (KAN), as a selection marker for mycobacteria. However, mutation in the single rRNA operon of slow-growing mycobacteria also causes resistance to KAN and thus limits the application of this selection marker (Böttger, 1994; Hatfull, 1996). Therefore, Radford and Hodgson (1991) introduced the hygromycin (HYG) resistance gene as a selection marker for mycobacteria. Their study revealed that the transformation efficiency was higher (10^3–10^5 transformants/μg DNA) when the vectors expressing the HYG resistance gene were used. Currently, HYG is a popular selection marker for *M. tuberculosis* without cross-resistance to anti-TB drugs (Garbe et al., 1994). Paget and Davies (1996) explored the potential of the apramycin resistance gene as a selection marker in both slow- and fast-growing mycobacteria. However, the apramycin resistance gene may confer resistance to two common antibiotics, KAN and gentamicin, which sometimes limits its utility in studies on mycobacteria (Consaul and Pavelka, 2004). Furthermore, chloramphenicol acetyltransferase gene conferring resistance to chloramphenicol, has also been used as a selection marker in some mycobacteria (Das Gupta et al., 1993); however, its application is limited owing to spontaneous mutation and poor stability. In addition, streptomycin (STR), sulfonamide (Gormley and Davies, 1991), mercurysalts (Baulard et al., 1995), gentamicin (Parish et al., 2007) and thiostrepton (Mugweru et al., 2017) have been reported as possible selection antibiotics in mycobacterial research.

3. Mutagenesis methods

3.1. Transposon mutagenesis

Transposons are mobile genetic elements that insert themselves randomly or at specific positions within the genome of an organism. Insertion of these mobile genetic elements into a target gene alters its expression and consequently, the resultant phenotype (Fig. 2). Many transposable elements have been identified in mycobacteria, with IS6110 from *M. tuberculosis* being the most useful for transposon mutagenesis studies (Collins and Stephens, 1991; Guilhot et al., 1992). The first mycobacterial insertional mutant libraries were generated by Guilhot et al. (1994) who used temperature-sensitive plasmid to deliver transposon Tn611 into the *M. smegmatis* chromosome, and the transposon was inserted at different locations with a high degree of randomness. The major limitation of this technique was inability of plasmid excision from the genome and its elimination from mycobacterial cells owing to its thermosensitive nature. This shortcoming was successfully addressed by using a bacteriophage to deliver the transposon into the *M. tuberculosis* chromosome; this bacteriophage-based approach was very efficient and more mutants were generated (Bardarov et al., 1997). Subsequently, independent research groups generated transposon mutant libraries in different strains of *M. tuberculosis* by using IS1096, Tn5370, Tn552, MycoMarT7, and Himar1 transposon systems (Camacho et al., 1999; McAdam et al., 2002; Darwin et al., 2003; Lamichhane et al., 2003; Shimono et al., 2003; Pethe et al., 2004; Rengarajan et al., 2005; Hernandez-Abanto et al., 2007; Dutta et al., 2010). A complete and detailed insertion mutant library of *M. tuberculosis* H37Rv was constructed by using a Tn5370 transposon system (McAdam et al., 2002). In the study, 1474 insertion mutants were identified among which 1329 insertion sites were found to be unique, and 116 and 13 insertion sites were found in the same position twice and thrice, respectively. Only one insertion site was identified in the exact same position four times, and 351 ORFs of *M. tuberculosis* H37Rv were disrupted (McAdam et al., 2002). Subsequently, Lamichhane et al. (2003) generated 1425 insertion mutants in *M. tuberculosis* using Himar1 transposon. Their study revealed 35% of potentially essential genes present in the genome of *M. tuberculosis* and seven functional groups were found to enrich in essential genes. To date, more than 4300 defined transposon insertion mutants in *M. tuberculosis* have been collected through Tuberculosis TARGET programme (http://webhost.nts.jhu.edu/target). Mutations obtained by aforementioned transposon systems warrant further validation via screening and *in vivo* analysis experiments using mice and macrophage infections, which is a lengthy process. Sassetti and coworkers (2003) introduced the transposon site hybridization (TraSH) strategy in *M. tuberculosis* to identify the genes required for optimal growth, infection of mice and survival in macrophages. Additionally, Beste et al. (2009) identified the genes required for slow- and fast-growth on minimal media using the TraSH method. Transposon insertion mutagenesis combined with deep sequencing has been used to identify protein-coding genes as well as other genomic elements, including non-coding RNAs which have important roles in optimal growth (Griffin et al., 2011; Zhang et al., 2012). This approach was successfully implemented in the identification of genes responsive to acidic stress and nitric oxide, genes responsible for survival in the host dendritic cells, and genes that play important roles in infection (Darwin et al., 2003; Vandal et al., 2008; Mendum et al., 2015; Barczak et al., 2017). The approach has also been used to identify different *M. tuberculosis* functional networks, such as those responsible for antibiotic tolerance and those protecting the bacteria from oxidative stress

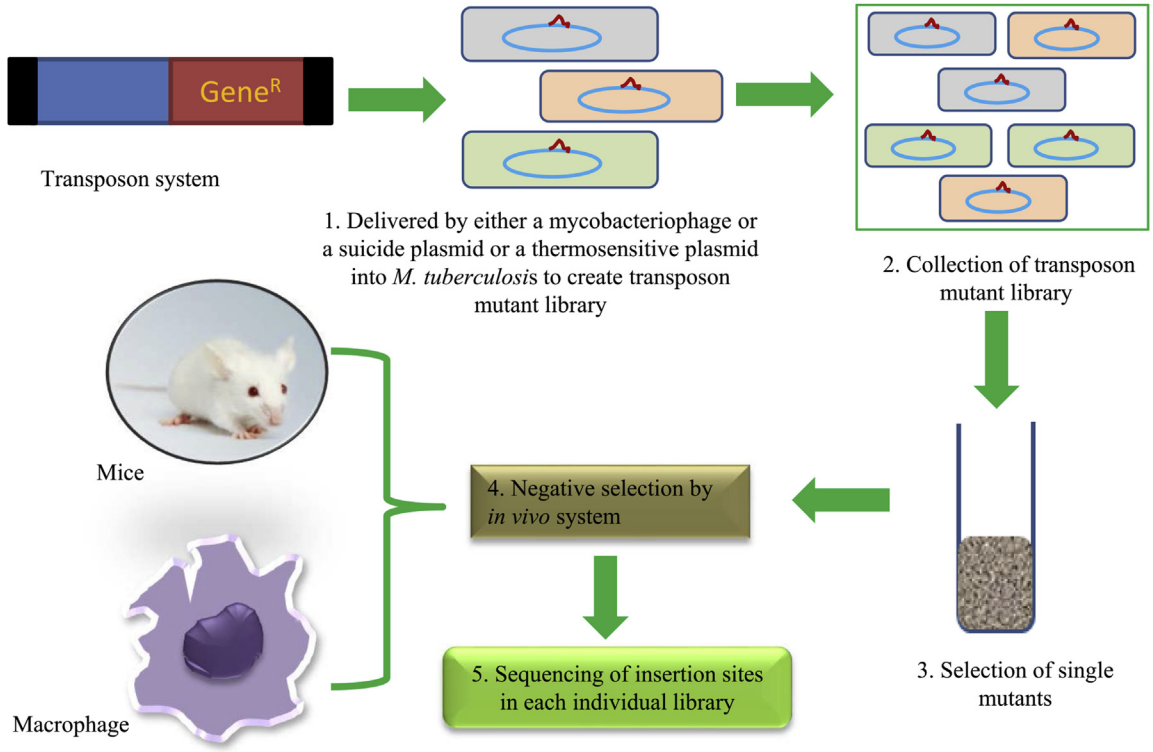

Fig. 2. Transposon mutagenesis in *M. tuberculosis*. The transposon system is delivered into *M. tuberculosis* via mycobacteriophage or suicide plasmid or thermosensitive plasmid to generate a transposon mutant library. All the transposon mutants are assembled into a library and single clones are identified. Thereafter, the mutants are used to infect either mice or macrophages. The insertion sites of each library are sequenced via high-throughput sequencing to identify the mutants. Gene^R represents resistance gene.

during infection and the pupylated proteasome substrate which protects bacteria from host-produced nitric oxide (Mestre et al., 2013; Kieser et al., 2015; Nambi et al., 2015; Samanovic et al., 2015). Dutta et al. (2014) extrapolated *M. tuberculosis* genes responsible for long-term survival in mouse lung using computational algorithms. More recently, DeJesus et al. (2017) analyzed a completely saturated transposon library for *M. tuberculosis* and generated a comprehensive catalog of *in vitro* essentiality, encompassing ORFs smaller than previously reported ORFs, small non-coding RNAs (sRNAs), promoters, and other genomic features. Despite its numerous potential applications, transposon mutagenesis approach is primarily limited by the polar effect of the genes owing to random insertion and the lack of a standardized effective method in mutant analysis.

3.2. Signature-tagged mutagenesis

Signature-tagged mutagenesis (STM) is an advanced traditional transposon mutagenesis method based on negative selection of mutants. STM is used to identify virulence genes, which facilitates the study of bacterial pathogenesis (Fuller et al., 2000; Hong et al., 2000; Sheehan et al., 2003). This approach minimizes the number of animals required for *in vivo* screening of numerous mutants, thereby rendering it advantageous to random or traditional gene inactivation tools. In addition to transposons, DNA sequence tags, acting as bar codes, are used in STM (Saenz and Dehio, 2005; Mazurkiewicz et al., 2006) (Fig. 3). STM was first applied for the identification of the virulence genes in *Salmonella typhimurium* (Hensel et al., 1995). Later, STM was used to study the important roles of lipids in pathogenesis (Camacho et al., 1999; Cox et al., 1999), to identify the counter immune mechanism (Hisert et al., 2004) in *M. tuberculosis*, and to understand the molecular mechanisms of phagosome maturation arrest by *M. tuberculosis* in the host (Hisert et al., 2004; MacGurn and Cox, 2007). Rosas-Magallanes et al. (2007) used this approach in combination with *in vivo* models to identify novel *M. tuberculosis* genes (ABC transporter-encoding genes *Rv0986* and *Rv0987*) involved in the parasitism of human macrophages. Additionally, Dhar and McKinney (2010) reported that the inactivation of *cydC* encoding an ATP-binding cassette transporter subunit enhanced the clearance of bacteria in isoniazid (INH)-treated mice, without influencing their growth. However, disruption of the *Rv0096-Rv0101* gene clusters resulted in the slow bacterial growth and protected *M. tuberculosis* in INH-treated mice. Despite all the above-mentioned advantages, the application of STM is limited to finding non-essential genes in bacteria. This approach involves secondary screening to reduce the number of false-negative candidates and requires the use of animal models, which is time-consuming.

3.3. Mutagenesis based on allelic replacement systems

3.3.1. Gene replacement using short and long linear DNA substrates

Chromosomal gene inactivation is the principal approach used in bacterial genomic functional studies. Homologous recombination (HR), a mechanism generally used by pathogens to maintain genomic stability, has been widely adopted to study gene function by inactivating the chromosomal genes in mycobacteria and in other bacteria. Successful disruption of *M. tuberculosis* genes, using short and long linear DNA fragments as allelic exchange substrates for HR has been reported (Balasubramanian et al., 1996; Armitige et al., 2000; Piddington et al., 2001). These linear DNA substrates carrying the target gene as well as a selection marker (*kan*^{R} and/or *hyg*^{R}) were transformed into *M. tuberculosis* via electroporation to disrupt the target gene. Short linear substrates (<5 kb) have been widely used to generate target gene mutants in different strains of *M. tuberculosis* (Kalpana et al., 1991; Berthet et al., 1998; Yuan et al., 1998; Armitige et al., 2000; Piddington et al., 2001). Only one study used 40–50 kb long linear DNA substrates to disrupt *leuD* gene in *M. tuberculosis* (Balasubramanian et al., 1996). Even though the technique uses high amounts of substrate, low transformation efficiency coupled with low HR rates in *M. tuberculosis* limits its use.

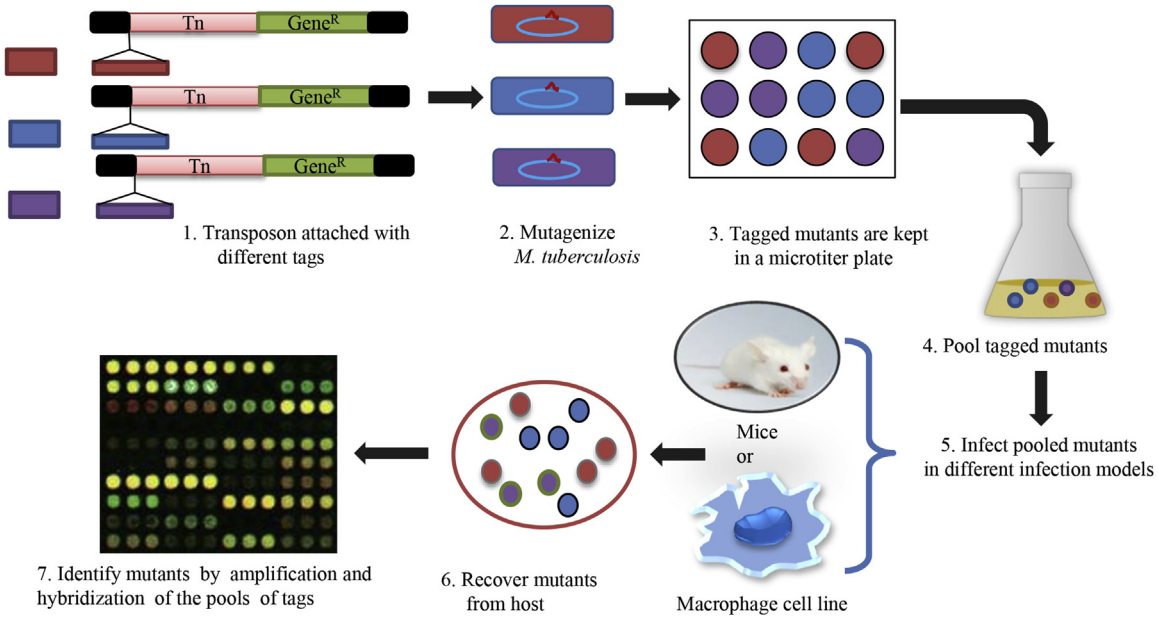

Fig. 3. Signature-tagged mutagenesis in *M. tuberculosis*. Pools of different tags (indicated as colored squares) are attached to the transposons. Thereafter, tagged transposons are delivered into *M. tuberculosis* and tagged mutants are maintained in microtiter plates as a library. Entire tagged mutants are pooled out to infect mice or macrophages. The bacteria are recovered from the host infected tissue or macrophages and plated out. The mutants are identified by amplifying and hybridizing the pools of tags.

3.3.2. Gene replacement via UV irradiation

UV irradiation involves the induction of a mutation in the target gene via UV treatment before electroporation into *M. tuberculosis*. Using the method and single-stranded phasmid DNA, Hinds et al. (1999) disrupted the putative haemolysin *tlyA* gene in *M. tuberculosis*. Similar observations were reported by Parish et al. (1999 and 2000) and Gordhan et al. (2002) used a similar approach to replace amino acid biosynthesis and phospholipase genes in *M. tuberculosis*. Subsequently, Singh et al. (2005) studied the role of the *mymA* operon to protect *M. tuberculosis* against adverse conditions by using this method. UV irradiation is considered the simplest approach implemented to obtain a large number of transformants. However, the possibility of secondary mutations generated in the *M. tuberculosis* genome by the DNA-damaged vector used for recombination is one of the major factors that may lead to failure in achieving gene replacement by this approach.

3.3.3. The counter-selectable marker approach

The construction of unmarked mutant bacteria, wherein a gene is replaced by a genetic marker, is a fundamental approach used to understand the bacterial pathogenicity at the molecular level. Counter-selectable markers are robust bacterial genetic tools facilitating the loss of a genetic marker and selection of recombination products while serving as reporter genes (Reyrat et al., 1998). The *sacB* marker has been widely used in *M. tuberculosis* for positive selection of recombination events. *sacB* gene from *Bacillus subtilis* encodes levansucrase catalyzing the hydrolysis of sucrose and synthesizing levans, which are toxic to mycobacteria. The expression of *sacB* in the presence of sucrose is lethal to mycobacteria (Pelicic et al., 1996). Consequently, Pelicic et al. (1997) designed ts-*sacB* vectors with the combination of the thermosensitive origin of replication and *sacB* as the counter-selectable marker used for knockout of mycobacterial genes. Additionally, the mutants can be efficiently counter-selected in sucrose at 39°C. It has been well adopted for generation of marked and unmarked *M. tuberculosis* mutants (Wolschendorf et al., 2011; Yang et al., 2014; Gutka et al., 2015; Villela et al., 2017). The use of this counter-selectable marker facilitates positive selection of double recombinants and enhances allelic exchange in the presence of sucrose; however, it is limited by the high frequency of spontaneous mutations (Muttucumaru and Parish, 2004). Similarly, the counterselectable marker *rpsL* has been used extensively for gene replacement in *M. tuberculosis* (Sander et al., 1995; Springer et al., 2001b; Raynaud et al., 2002). This system is based on a vector-host system, wherein a wild-type *rpsL* gene encoding ribosomal protein S12 is expressed in a STR-resistant host. It uses the KAN-resistance gene and a STR-sensitive allele of *rpsL* for selection. However, its utility in genetic studies is limited by the need for a STR-resistant background strain and STR is a second-line anti-TB drug. In addition to *sacB* and *rpsL, E. coli*-derived *galK* gene encoding galactokinase has been used for gene replacement in *M. tuberculosis* (Barkan et al., 2011). Galactokinase phosphorylates galactose to galactose-1-phosphate, and 2-deoxy-galactose (2-DOG, a galactose analog) to 2-deoxy-galactose-1-phosphate. The second product 2-deoxy-galactose-1-phosphate cannot be metabolized and accumulates to toxic levels that are lethal to *M. tuberculosis* cells. The construction of the unmarked mutants of *M. tuberculosis* is achieved through insertion of *galK* in the target gene within the genome and then incubating the transformed cells in medium containing 2-DOG.

Despite the fact that counter-selectable markers have been successfully used for the construction of unmarked mycobacteria, the genetic instability due to the low level dissociation by intgrase and lengthy two-step selection limit their utility. To address the "two-step" issue, Xer-cise technique introduced in mycobacteria for the construction of selectable marker-free recombinant strains (Cascioferro et al., 2010) in one step. Later, Yang et al. (2014, 2015) engineered *dif* -ΩHYG-*int-dif* cassette which can be excised efficiently by Xer-site-specific recombination system of mycobacteria in one step and the resulting selectable marker-free recombinant mycobacterial strains are very stable.

3.3.4. Phage-based gene knockout systems

The success rate of gene replacement by HR depends on efficient gene transfer in mycobacteria, which is a major challenge. In this regard, hybrid shuttle phasmids, consisting of mycobacteriophage DNA and *E. coli* cosmid capable of replicating in *E. coli* as plasmids and in mycobacteria as phages, have been introduced (Jacobs et al., 1987; Bardarov et al., 1997; Cox et al., 1999). This system inserts foreign genes into the mycobacterial genome mainly by HR or transposon. An early attempt to introduce a foreign DNA into *M. smegmatis* and *M. bovis* BCG using a shuttle phasmid was made by Jacobs et al. (1987). Subsequently, conditionally replicating shuttle phasmids were used to generate transposon mutant libraries in *M. tuberculosis* (Bardarov et al., 1997; Cox et al., 1999). The system was modified to generate gene mutations in *M. tuberculosis* through *in vitro* generated specialized transducing mycobacteriophages (Bardarov et al., 2002). In recent years, Jain et al. (2014) improved the cloning capacity of phAE159 shuttle phasmid to harbor larger DNA fragments. This shuttle phasmid replicates as a phage at 30°C and remains quiescent at 37°C. Furthermore, Tufariello et al. (2014) combined recombineering and phage-based transduction methods to efficiently generate targeted *M. tuberculosis* mutants. This specialized transduction system has been successfully been implemented for the generation of gene mutations in different strains of *M. tuberculosis* in the recent past years (Williams et al., 2011; Forrellad et al., 2013; Berney et al., 2014; Kandasamy and Narayanan, 2015; Olsen et al., 2016; Gomez et al., 2016; Korte et al., 2016; Khan et al., 2017). The major drawbacks of this technology are the tedious steps required to obtain a single mutant strain coupled with the difficulty in excision of the counter-selectable marker from the recombinants. Additionally, the efficacy of this approach is dependent on *in vitro* packaging kits which are expensive and inconvenient to use.

3.3.5. Recombineering system

Recombineering is based on the expression of phage proteins that enhance the HR rate between target genomic sequences and exogenous DNA fragments (either dsDNA or ssDNA). This method was first reported and designated by Yu et al. (2000) to modify the *E. coli* genome. Thereafter, Van Kessel and Hatfull (2007) used the GP60 and GP61 proteins from mycobacteriophage Che9c to manipulate the mycobacterial genome. Che9c-GP60 possesses exonuclease activity, while Che9c-GP61 is a single-stranded DNA-binding protein (Fig. 4). These mycobacteriophage proteins are expressed in *M. tuberculosis* via acetamide-inducible plasmid pJV53. Additionally, the study showed that GP60 as well as GP61 is toxic to *M. tuberculosis*. However, when the proteins were co-expressed, the toxic effect was minimized. Concurrently, Van Kessel and Hatfull (2008) reported the introduction of a single point mutation in the *M. tuberculosis* chromosome by using Che9c GP61-mediated recombineering. The development of this robust technique simplified gene replacement in *M. tuberculosis* as well as other mycobacterial species. Rcombineering method has been successfully implemented to knock out target genes of *M. tuberculosis*, despite its limitation of removal of the pJV53 plasmid from the cell (Gouzy et al., 2014; Le Chevalier et al., 2015; Palucci et al., 2016; Deboosère et al., 2017). However, according to the experiences from our and other labs, the successful rates using recombineering system in *M. tuberculosis* is not as high as that in *M. smegmatis*.

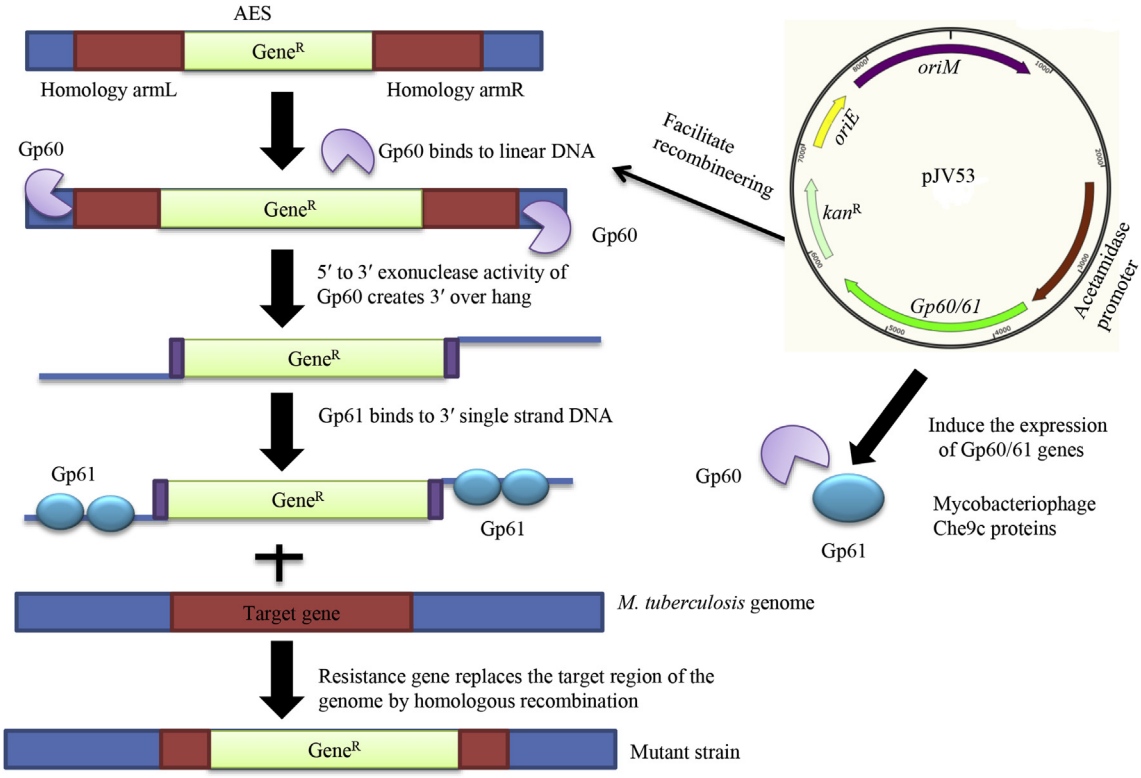

Fig. 4. Recombineering-mediated gene replacement in *M. tuberculosis*. The pJV53 plasmid contains *GP60*/*GP61* genes transcribed by an acetamide inducible promoter. GP60 protein degrades one DNA strand of the allele exchange substrate (AES) in a 5′ to 3′ direction, and GP61 single standard binding protein binds to ssDNA to prevent further degradation and facilitate single-strand annealing. Thereafter, homologous pairing and DNA strand exchange occur between the homologous arms. Finally, the target gene in the genome is replaced by the resistance marker gene. Gene^R represents resistance gene.

4. Inducible expression system

HR-based gene replacement approaches have been used for functional studies of target genes in *M. tuberculosis* for 20 years; however, it is impossible to disrupt an essential gene to study its function directly in *M. tuberculosis*. To overcome this limitation, Triccas et al. (1998) first established an inducible expression system by using regulatory elements derived from *amiE* encoding acetamidase when induced by acetamide in *M. smegmatis*. The system was utilized to investigate the function of essential genes in *M. smegmatis* (Greendyke et al., 2002); however, its application in *M. tuberculosis* was limited by genetic instability and the complexity of regulation (Parish et al., 2001; Roberts et al., 2003). The system was later replaced by the tetracycline (tc)-regulated expression system (Blokpoel et al., 2005; Ehrt et al., 2005; Carroll et al., 2005), which is regulated by a tc repressor protein (TetR) with high binding specificity towards *tet* operator (tetO) and regulates the expression of tc-resistant determinants in bacteria (Hillen and Berens, 1994; Berens, 2003). The implementation of tc-regulated expression system in mycobacteria was first reported by independent research groups in the year of 2005 (Carroll et al., 2005; Ehrt et al., 2005; Blokpoel et al., 2005). This system functions efficiently to regulate target gene expression in *M. tuberculosis in vivo* (Hernandez-Abanto et al., 2006). However, the major limitation of this system was the difficulty in removing tc from the culture to achieve regulation of target gene in mycobacteria efficiently. To overcome this limitation, Guo et al. (2007) used the mutated reverse TetRs (revTetRs) allowing efficient gene regulation and the construction of conditional mutants of target gene in *M. smegmatis*. This improved system was used to study the role of 20S proteasome in *M. tuberculosis* by regulating the expression α and β subunits gene *in vivo* (Gandotra et al., 2007). The system was further modified by optimizing the revTetR codons to achieve high level of gene regulation. Later, tc-regulated expression system was successfully implemented to study the role of target genes in *M. tuberculosis* (Carroll et al., 2011; Leblanc et al., 2012; Korte et al., 2016; Degiacomi et al., 2017), validating potential drug targets (Ollinger et al., 2012; Abrahams et al., 2012; Kolly et al., 2014; Singh et al., 2015) and the development of small molecule inhibitors (Wang et al., 2013; Moolman et al., 2014; Brecik et al., 2015).

Akin to the acetamide and tetracycline-inducible system, Forti et al. (2009) developed the pristinamycin-inducible expression system for mycobacteria. This system is based on the *Streptomyces coelicolor* pristinamycin-responsive protein (Pip) repressor which binds to the promoter of the pristinamycin (*ptr*) of *Streptomyces pristinaespiralis* to repress *ptr* transcription. The transcription repression depends on the presence of the inducer pristinamycin I and other antibiotics (Salah-Bey et al., 1995; Folcher et al., 2001). However, pristinamycin is toxic to *M. tuberculosis*; hence, Boldrin et al. (2010) developed a novel repressible promoter system based on the TetR and Pip repressors. In this system, Pip is tightly regulated by TetR in the absence of the anhydrotetracycline (aTc) inducer which facilitates the expression of a target gene present downstream of the *ptr* promoter. In the presence of aTc, Pip is expressed and induces the downregulation of the target gene under the *ptr* promoter. This dual regulatory system was implemented to regulate the expression of *fadD32*, an essential gene in *M. tuberculosis* (Boldrin et al., 2010).

The isopropyl β-D-1-thiogalactopyranoside (IPTG) -inducible expression system frequently used in *E. coli* relies on a promoter

repressed by LacI repressor and can be induced in the presence of IPTG (Terpe, 2006). Two independent research groups used the LacI regulated expression system to assess target gene functions in mycobacteria (Lee et al., 2008; Kaur et al., 2009). In the first study, an IPTG-inducible GFP expression system was introduced into *M. tuberculosis* by using LacI repressor which binds to the T7 RNA polymerase promoter (Lee et al., 2008) and was successfully used to study the metabolic activity of intracellular *M. tuberculosis*. In the second study, a lac operator was placed downstream of a mycobacterial promoter to facilitate repression by LacI repressor (Kaur et al., 2009). Ravishankar et al. (2015) developed the non-replicating IPTG-inducible vectors for mycobacteria and explored the effect of a single lac operator *versus* a double lac operator for regulating gene expression in *M. smegmatis*. The IPTG-inducible vector system was used to investigate the vulnerability of polyphosphate kinase and the inhibition effect of potential drug targets in *M. tuberculosis* (Jagannathan et al., 2010; Kaur et al., 2016). Recently, Refaya et al. (2016) used IPTG-inducible expression system to elucidate the role of the *PknL* gene in adaptive responses of *M. tuberculosis*. However, few studies on the use of this inducible system in mycobacteria have been reported. Therefore, this inducible expression system needs further exploration.

5. Conditional gene silencing approaches

5.1. Controlled proteolysis

Transcriptional gene silencing approach has been used for the identification and validation of potential drug targets and study the functions of target genes in *M. tuberculosis*. However, it is limited by its inability to regulate protein stability. To resolve this issue, Kim et al. (2011) developed a protein degradation machinery to inhibit the target proteins in mycobacteria. The system utilizes the SspB adapter protein to recognize proteins tagged with DAS+4 (AANDENYSENYADAS) and enhances their degradation by ClpXP proteases. In this approach, the gene for SspB adapter protein under the control of an inducible promoter is introduced into the mycobacterial genome, followed by the introduction of a plasmid harboring a sequence homologous to the 3′ end of target gene with a DAS+4 tag which is incorporated into the mycobacterium genome in a single crossover event. The target sequence expressed with the DAS+4 tag, is recognized by the SspB adapter in the presence of an inducer, allowing the degradation of the protein by ClpXP proteases. This method was successfully used to knock down essential genes of *M. tuberculosis*, including, *rpoB*, *clp P1*|*P2*, and *mmpL3* using a unique dual-control switch, resulting in proteolysis of the encoded protein (Kim et al., 2013; Raju et al., 2014; Li et al., 2016a, 2016b). Owing to its robustness, this method is a suitable candidate to identify the *M. tuberculosis* proteins essential for its growth and for non-replicating persistence *in vitro*; it can also be employed for identification of novel drug targets.

5.2. Antisense technology

Antisense technology facilitates the silencing of bacterial genes based on the principle of target ORF expression in the antisense orientation. Antisense sequences can hybridize to the target mRNA via multiple methods to inhibit target gene expression (Waters and Storz, 2009). The accuracy of this technique is constrained by the need for several standardizations to stabilize the secondary structure. In addition, the length of the antisense sequences, binding specificity, protection from intracellular nuclease activity, and availability of sufficient intracellular antisense RNAs are the technical restraints of this technology (Stach and Good, 2011). Parish and Stoker (1997) introduced antisense technology by overexpressing an antisense strand of *hisD* under an inducible promoter to study the growth of *M. smegmatis*. Subsequently, Wilson et al. (1998) used antisense RNA to inhibit *ahpC* expression in *M. tuberculosis* complex (MTBC) strains. Since then, the antisense technology has been adopted by other researchers to study the function of different genes in *M. tuberculosis* (Deol et al., 2005; Harth et al., 2007; Ahmed et al., 2014; Chandolia et al., 2014; Refaya et al., 2016; Kaur et al., 2016; Botella et al., 2017). In addition to antisense nucleotides, antisense peptide nucleic acids which are analogs of nucleic acids can be used to knock down the target gene expression because they form stable complexes with target DNA or RNA (Nielsen, 2004). They are not prone to nuclease and protease degradation, highly stable, and naturally hydrophobic (Demidov et al., 1994; Goh et al., 2014). This approach has been applied for the detection of MTBC in clinical samples (Stender et al., 1999), but not for gene silencing mycobacteria.

5.3. CRISPRi-mediated silencing

Various advanced genetic methods have been utilized to characterize the drug targets by regulating target gene expression in *M. tuberculosis*. However, targeting a single gene can be time-consuming and simultaneous regulation of multiple genes is a major challenge. The CRISPRi approach can overcome these methodological limitations. CRISPR/Cas9 approach has been extensively adopted for genome manipulation in present scenario (Mei et al., 2016). Jinek et al. (2012) developed the CRISPR/Cas9 system for genome editing. Later, this system has been used for gene silencing in several bacterial species (Qi et al., 2013; Mimee et al., 2015; Tong et al., 2015; Li et al., 2016a, 2016b). In CRISPRi system, catalytically inactive Cas9 (dCas9) is guided by the small sgRNA, and the whole complex binds to the promoter region or open reading frame of the target gene, which then represses the gene expression by inhibiting the initiation and elongation steps of transcription process (Fig. 5). Choudhary et al. (2015) and Singh et al. (2016) independently introduced CRISPRi system in mycobacteria. In their studies, dCas9 and target sgRNA were expressed in *M. tuberculosis* by the aTc-inducible promoter (Fig. 5). It was observed that conditionally expressed dCas9 in the presence of aTc is well tolerated in slow- and fast-growing mycobacteria, without a toxic effect on growth. Choudhary et al. (2015) used the CRISPRi technology to silence *engA*, *groEL1*, *yidC*, and other genes in *M. tuberculosis*. Singh et al. (2016) reported the knockdown of essential genes including Ser/Thr protein kinase, *inhA*, *dfrA*, *wag31*, and *ftsZ* in *M. tuberculosis*, and approximately 80% of gene expression was decreased by using this method. Subsequently, Rock et al. (2017) reported that how a dCas9 derived from *S. pyogenes* has low efficiency for target gene silencing besides showing the proteotoxicity in *M. tuberculosis*. To overcome these drawbacks, 11 Cas9 orthologus were screened and CRISPR1 Cas9 from *Streptococcus thermophilus* ($dCas9_{Sth1}$) was found to efficiently silence target genes in *M. tuberculosis* with minimum toxic effect. This robust technology may facilitate the study of *M. tuberculosis* pathogenicity. Recently, Singh et al. (2017) knocked down *Rv2672* using CRISPRi and investigated its role in the growth of *M. tuberculosis* under hypoxic stress. However, there are two major drawbacks of the CRISPRi approach: one is the off-target effect and the other is the likelihood of polar effect on the expression of genes located at the 3′ end of the same operon when CRISPRi target gene is present at the 5′ end of a polycistronic operon. However, a CRISPR system that used to effectively knock out genes in *M. tuberculosis* has not been reported yet.

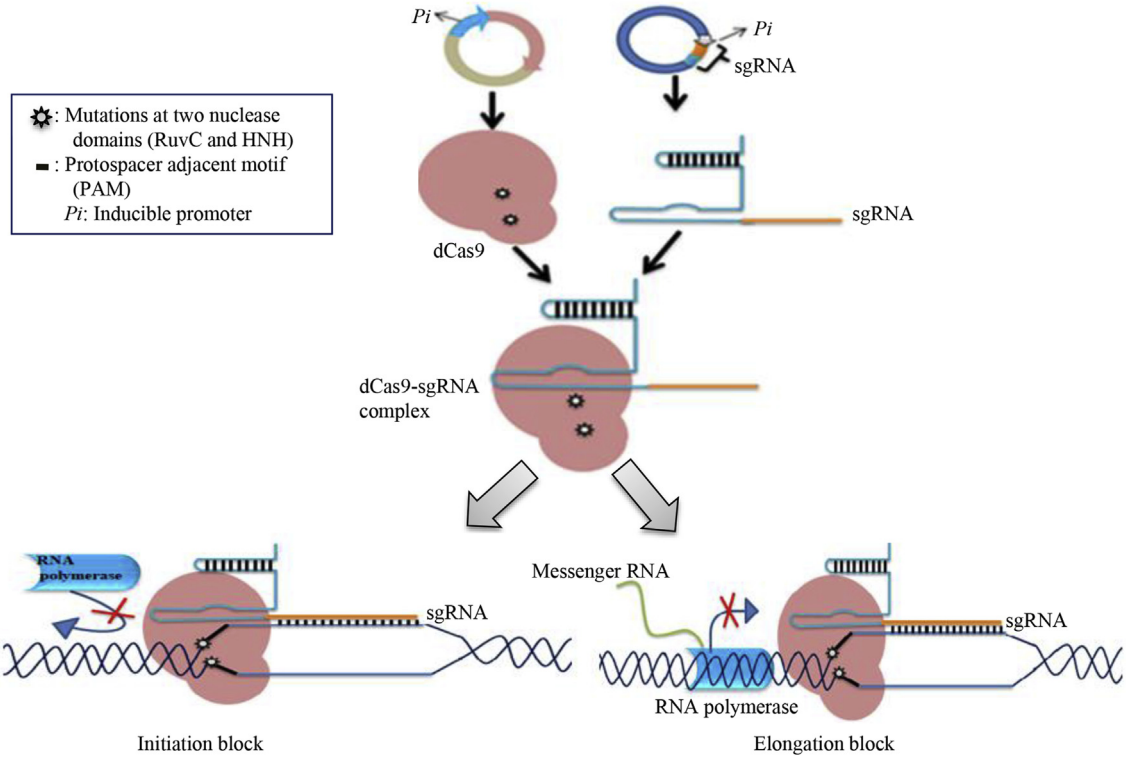

Fig. 5. CRISPRi mediated gene silencing in *M. tuberculosis*. Target small guide RNA (sgRNA) and dCas9 are co-expressed by aTc inducible promoter. sgRNA is recognized by dCas9 to form a protein-RNA complex. The complex binds to the sequence in the chromosome near the protospacer adjacent motif (PAM) by complementary base pairing between the target-specific sequence of sgRNA (orange color) and target sequence in the chromosome. This blocks either the initiation or elongation step of transcription.

6. Clinical applications of molecular genetic tools

6.1. Whole-genome sequencing

The use of genomic and bioinformatic tools has enabled the establishment of platforms to study new therapies and antimicrobial drugs geared towards the eradication of this airborne pathogen. The importance of whole-genome sequencing (WGS) technology in mycobacterial research was appreciated when Cole et al. reported the first genome sequence of *M. tuberculosis* strain H37Rv in 1998 (Cole et al., 1998), which improved our understanding of its genetics, pathogenicity, physiology, and metabolism. With a genome size of 4,411,529 bp and a G+C content of 65.6%, *M. tuberculosis* H37Rv has 3924 open reading frames (ORFs) and the genome is rich in repetitive DNA sequences, multi-gene families, and duplicated housekeeping genes. The *M. tuberculosis* genome harbors 50 genes that encode functional RNAs; the genes encoding tRNAs recognize 43 of the 61 possible sense codons. Subsequently, two 10-kb prophages (phiRv1 and phiRv2) were identified from the genomes of *M. tuberculosis* strains H37Rv and CDC1551 (Bibb and Hatfull, 2002; Bibb et al., 2005). NGS technology provides accurate, large-scale genomic information, as it is less time-consuming and more cost-effective (Metzker, 2010). In recent years, the NGS approach has been used in various clinical scenarios, including the prediction of drug susceptibility, diagnosis of TB, and genetic diversity studies (Satta et al., 2017). The application of NGS in *M. tuberculosis* was first reported by Andries et al. (2005), who identified ATP synthase as the target of diarylquinoline, a novel anti-TB drug by using 454 pyrosequencing. This was followed by WGS of XDR strains from South Africa (Ioerger et al., 2009). Using the WGS technology, Casali et al. (2014) analyzed the genomes of 1000 M. *tuberculosis* drug-resistant isolates from Russia and correlated the gene mutations to their corresponding phenotypes. Additionally, 28 intergenic regions (IGRs), 72 new genes, 11 non-synonymous single-nucleotide polymorphisms (SNPs), and 10 IGR SNPs were associated with drug resistance, as identified via sequencing and an analysis of 161 clinical isolates from China (Zhang et al., 2013). Similar studies revealed the association of uncharacterized genes, intergenic regions, and SNPs with *M. tuberculosis* drug resistance phenotypes (Anderson et al., 2014; Ali et al., 2015; Pankhurst et al., 2016). NGS technology has also been applied to obtain drug resistance profiles directly from clinical isolates, which facilitates to reduce the treatment duration of patients (Galagan, 2014). A recent proof-of-concept study by Colman et al. (2016) reported drug resistance profiles directly from clinical samples, using tabletop NGS technology. Furthermore, NGS technique has been used in the development of clinical diagnostic tests because it can provide bacterial whole genome sequences in a very short duration (hours to days) at very low cost and without requirement for bacterial cultures (Bjorn-Mortensen et al., 2015; Witney et al., 2016). The depth of whole genome sequence information provided by NGS has accelerated the accuracy and speed of MDR/XDR-TB diagnosis and the epidemiological investigation of TB (Roetzer et al., 2013; Luo et al., 2014; Jeanes and O'Grady, 2016). Similarly, NGS technology has been used for comparative genomic analysis, which provides information about genomic diversity and evolution of drug-resistant *M. tuberculosis* (Liu et al., 2014). Eldholm et al. (2014) initially attempted to study the evolution of XDR-*M. tuberculosis* strains from a susceptible ancestor in the same patient. Similarly, Zhang et al. (2016) reported the evolutionary correlation between fluoroquinolones-sensitive and -resistant *M. tuberculosis* strains isolated from a single patient using NGS technology. Recently, Wollenberg et al. (2017) elucidated the genetic composition and evolution of 138 MDR- and XDR-

M. tubercolosis isolates in Eastern Europe. Although WGS analysis yields results more quickly than standard drug susceptibility tests (DSTs); however, it is limited by cumbersome data analysis and the data inconsistencies between the phenotypic DST and gene mutations.

NGS has been successfully used to define the *M. tuberculosis* genome; however, more accurate, complete, and rapid DNA sequencing technologies are required. The emergence of third-generation sequencing has enabled the production of long reads with an average size range of 5–15 kb with some reads being as long as 100 kb (Lee et al., 2016). These long reads span most repeat sequences and thus enable closure of the gaps in fragmented assemblies; this facilitates the assembly of complex genomes and simplifies *de novo* assembly of small microbial genomes (Roberts et al., 2013). Third-generation sequencing also facilitates the detection of base modifications (methylation and phosphorothioate) in genomes (Flusberg et al., 2010). Three commercially available third-generation sequencing platforms are Pacific Biosciences (PacBio) Single Molecule Real Time (SMRT) sequencing, the Oxford Nanopore Technologies MinION, and Illumina Tru-seq Synthetic Long-Read technology (Lee et al., 2016). Recently, several bacterial whole genome sequences together with methylomes have been reported using this new sequencing technology (Fang et al., 2012; Krebes et al., 2014; Fomenkov et al., 2017; Zautner et al., 2017). In 2016, Zhu et al. sequenced the whole genomes of 12 MTBC strains and characterized their methylomes by using SMRT sequencing. Three m^6A motifs and their corresponding DNA MTase genes in the *M. tuberculosis* genomes were identified. It was revealed that MTases activities varied within different MTBC lineages. Similarly, SMRT technology has been used to identify mutations that contribute to stepwise evolution of drug resistance in MDR-TB clinical isolates derived from the same patient (Leung et al., 2017). In another study, Jia et al. (2017) investigated the relationship between the genotype and phenotype of 12 MTBC strains. Rodríguez-Castillo et al. (2017) used SMRT technique to demonstrate that specific genetic variations were associated with mycobacterial virulence and drug resistance. Elghraoui et al. (2017) re-sequenced the *M. tuberculosis* H37Ra genome by using the SMRT sequencing technology and found that the number of H37Ra-specific SNPs is less than half of that in a previously reported H37Ra reference sequence. Recently, Phelan et al. (2018) analyzed the methylomes across the four major lineages of *M. tuberculosis* and identified lineage-specific methylation motifs and key mutations in the associated genes, which were found to be globally distributed. Furthermore, genomes of several *M. tuberculosis* clinical isolates have been sequenced using third-generation sequencing platforms (Rodríguez et al., 2015; Philip et al., 2016; Wada et al., 2017a, 2017b). Despite the robustness of third-generation sequencing, the cost is higher than that of the second-generation technologies. Additionally, the error rate of a single read of the third-generation sequencing is around 15%–40%, and the sequencing requires high coverage of approximately 30 × to 70 ×, which limit its applications for the study of more mycobacterial genomes.

6.2. Mycobacteriophage system

The increased drug resistance in *M. tuberculosis* is a major concern for TB control. The use of mycobacteriophages is an alternative approach, which when put into clinical use, could open a new window on TB research (McNerney and Traore, 2005). Mycobacteriophages are the viruses that infect mycobacteria. In 1946, mycobacteriophages were first discovered to infect *M. smegmatis* (Gardner and Weiser, 1947). Later, mycobacteriophages were found to infect *M. tuberculosis* by Froman et al. (1954). Currently, studies are being undertaken for the development of mycobacteriophage-based tools for therapeutic and diagnostic purposes and for drug resistance detection. Owing to their ability to lyse and kill bacteria, mycobacteriophages have been put to use in the treatment of various infectious diseases. Decades ago, phage therapy was used in many countries for treatment of bacterial diseases (Sulakvelidze et al., 2001). The use of phage therapy to treat TB in animal was first attempted in 1963 (Hauduroy and Rosset, 1963; Mankiewicz and Beland, 1963). Unfortunately, treatment with mycobacteriophages did not cure the infected experimental animals and had a negative effect on their growth. Sula et al. (1981) observed that DS-6A phage therapy reduced lesions in the spleens, lungs, and livers of guinea pigs infected with *M. tuberculosis*. Similarly, Zemskova and Dorozhkova (1991) confirmed the therapeutic action of DS-6A phage against disseminated TB in guinea pigs. However, the curative effect was not comparable to that of INH monotherapy. Although mycobacteriophages could be used for therapeutic purposes, killing the intracellular *M. tuberculosis* presenting in the macrophage was a major challenge. Additionally, the phage needs to cross the mammalian cell membrane and survive in the intracellular acidic environment. To overcome these limitations, Broxmeyer et al. (2002) used *M. smegmatis* as a carrier to transport TM4 bacteriophage into macrophages. Interestingly, the infection was significantly reduced in both *M. tuberculosis*- and *Mycobacterium avium*-infected macrophage cell lines. Samaddar et al. (2015) reported that D29 mycobacteriophage could kill *M. smegmatis* through lysis and the infected cells produced toxic superoxide. One existing technical barrier for successful phage therapy is the presence of granulomas which may prevent the entry of phages into macrophages (Bowman et al., 1972). Although treatment with lytic phages rapidly eliminates mycobacteria *in vivo*, superantigens may be released and could enhance the inflammatory response. This could cause significant illness as a side effect of mycobacteriophage therapy.

Mycobacteriophages have also been used in diagnostic tests to detect *M. tuberculosis* in clinical samples and to assess drug susceptibility (Fu et al., 2015). Mycobacteriophage technology was first introduced for clinical use by Wilson et al. (1997). Mycobacteriophage D29 is commonly used for the detection of MTBC because it can infect slow growing mycobacteria such as *M. tuberculosis* and *M. ulcerans* (David et al., 1980; McNerney, 2001). A highly sensitive mycobacteriophage was developed by McNerney et al. (1998) to identify viable *M. smegmatis* and *M. tuberculosis*. Subsequently, McNerney et al. (2004) used D29 mycobacteriophage replication assays to identify *M. tuberculosis* from 496 sputum specimens with 44.1% and 92.6% of sensitivity and specificity, respectively. Using this technology, two commercial kits were developed by Biotec Laboratories Ltd. (Ipswich, UK) for rapid TB diagnosis and analysis of antibiotic susceptibility (Mole and Maskell, 2001; Seaman et al., 2003). The efficacy of this assay was evaluated to detect *M. tuberculosis* from clinical isolates in multiple countries, including Pakistan (Muzaffar et al., 2002), South Africa (Albert et al., 2002), Egypt (Marei et al., 2003) and Spain (Alcaide et al., 2003). Moreover, a reporter phage was also engineered for TB diagnosis and analysis of drug susceptibility. Jacobs et al. (1993) developed luciferase reporter mycobacteriophages (LRPs). The firefly luciferase (*FFlux*) gene was inserted into phAE39 shuttle plasmid and expressed under the strong promoter of heat shock protein 60 (*hsp60*). These LRPs can detect low amounts of *M. tuberculosis* (~10^4/mL) in clinical samples within a few minutes post infection. Other LRPs have been constructed based on L5 (Sarkis et al., 1995) and D29 (Pearson et al., 1996). However, the lytic properties of certain phages (D29 and TM4) reduce light output and sensitivity. Additionally, the efficiency of L5 to infect *M. tuberculosis* is very low and thus its application to detection of drug-resistance in clinical isolates is

limited. To overcome these limitations, Kumar et al. (2008) modified the LRPs by using Che12 temperate phage, resulting in sustained light output and improved detection of drug resistance. However, these LRPs are able to detect only viable *M. tuberculosis* from clinical samples and not dormant bacteria. To increase the efficiency of detection of dormant or non-active *M. tuberculosis* by LRPs, Dusthackeer et al. (2008) used promoters of *hsp60*, isocitrate lyase, and alpha crystallin genes to express the firefly luciferase gene in two mycobacteriophages (TM4 and Che12). Compared to previously used LRPs, the modified LRPs increased the light output in tested clinical isolates of *M. tuberculosis* H37Rv. These modified LPRs were used by Dusthackeer et al. (2012) to detect tubercle bacilli directly from sputum samples. Importantly, Banaiee et al. (2008) evaluated the drug susceptibility of clinical isolates against two anti-TB first-line drugs, using modified LPRs and compared their efficiency with that of BACTEC 460 radiometric mycobacterial broth culture system assay. The results of the study revealed that specificity and sensitivity of LRPs for drug susceptibility detection are nearly the same as those of the BACTEC 460 assay. However, LRPs assay consumes less time (2 days). Another group of reporter mycobacteriophages used for clinical purposes are the fluoromycobacteriophages. The first fluoromycobacteriophages were constructed by Piuri et al. (2009) using fluorescent reporter genes *gfp* or *ZsYellow*. This technique does not require any substrate for light output and can detect cells even when less than 100 mycobacterial cells are present. Further, this approach identified *M. tuberculosis* drug-resistant strains from mixed populations and detected drug susceptibility within 24 h with high specificity and sensitivity. Later, Rondón et al. (2011) engineered phAE87:hsp60-EGFP-enhanced green fluorescent protein (EGFP; EGFP-phage) based on TM4 mycobacteriophage harboring the gene encoding EGFP. EGFP-phages were used to determine drug resistance in 155 M. *tuberculosis* strains to INH, rifampin and STR. The results revealed that EGFP-phage had a sensitivity of 94% for all antibiotics and specificities of 90%, 93%, and 95% for INH, rifampin and STR, respectively. This approach is viable for detection of tubercle bacilli and rapid screening of drug resistance. However, it should be economically viable and more sensitive to detect MDR or XDR-TB. Additionally, the EGFP-phage infects and lyses bacteria, resulting in termination of EGFP expression, which affects sensitivity. To overcome this shortcoming, da Silva et al. (2013) designed a new reporter phage by inserting Phsp60-egfp cassette into the D29 mycobacteriophage genome for detection of mycobacteria. The fluoromycobacteriophage technology requires several modifications for diagnostics and drug susceptibility determination.

7. Conclusions and future perspectives

Owing to the great clinical relevance of this mycobacterial species, numerous studies have focused on its genetics. In this review, we describe various molecular approaches, tools and methods which are useful in studying mycobacterial genetics to investigate the mechanisms underlying pathogenesis and drug resistance, especially in *M. tuberculosis*. However, these parameters are deterred by the slow growth, spread of resistant strains, the rigid cell wall structure, and lack of efficient transformation tools in *M. tuberculosis*. Nonetheless, NGS technology has accelerated the prediction of drug-resistant strains and the determination of genetic relatedness. More importantly, the third-generation sequencing has provided ample *M. tuberculosis* genome and can be used to explore the role of the mycobacterial epigenome in antibiotic stress. However, large-scale implementation of modern WGS technologies require advanced software and suitable data analysis platforms. The clinical applications of the mycobacteriophage-based system are currently underway. Unfortunately, the mycobacteriophage therapy has not been successful in experimental animals; therefore, alternative strategies are required to develop efficient phage delivery systems to eliminate intracellular tubercle bacilli. Additionally, the mycobacteriophages are not able to detect dormant *M. tuberculosis*. Further studies are required to make them more sensitive and explore their utility in detecting smear-negative TB. In addition, the recombineering method developed by Van Kessel and Hatfull for *M. tuberculosis* (2007) is a popular method used to generate mutants; however, it is still time-consuming and success rates are not high in *M. tuberculosis*. The introduction of allelic exchange substrates and mycobacteriophage proteins (Gp60 and Gp61) into mycobacteria via a single vector system is an alternative strategy that will be more suitable for target gene deletion. The more recent CRISPRi approach has great potential in mycobacterial research; however, the applications of this method are curtailed by off-target effects. Importantly, Yan et al. (2017) reported the Cpf1 (CRISPR-Cas12a) system can knock out target genes in *M. smegmatis* very efficiently. Further studies are required to investigate the applications of the catalytically active Cas9 and CRISPR-Cas12a system for genome editing in *M. tuberculosis*. Moreover, the implementation of the CRISPR-Cas12a system for genome editing in mycobacteria would be more convenient and reliable than the CRISPR/Cas9 system. This system has the following advantages: (i) Cpf1 is guided by a short crRNA without tracrRNA, which will expand the possibility of multiplex genome editing in mycobacteria via expression of multiple crRNAs from a single plasmid; (ii) Cpf1 makes double strand-breaks at target sites that form sticky ends, leading to the deletion of 6–13 base pairs in contrast to Cas9 that generates deletion or addition of 1–3bp; (iii) Cpf1 has higher specificity and lesser off-target effects than Cas9. Additionally, DNase-dead Cpf1 (ddCpf1) can be used for gene regulation in *M. tuberculosis* in a similar manner to dCas9. The utility of CRISPR/Cas9 is not only limited in genome editing in mycobacteria, it can also be used as an antimicrobial agent against drug-resistant *M. tuberculosis* strains. In recent years, this concept has been reported in some bacterial species (Bikard et al., 2014; Ando et al., 2015; Yosef et al., 2015; Park et al., 2017). Additionally, Zhang et al. (2017) used CRISPR/Cas9 system to detect *M. tuberculosis* DNA with high specificity and sensitivity. Future studies are required to explore the applications of this technique to target drug-resistant strains of this pathogen in a sequence-specific manner and to resensitize the MDR and XDR strains, which will be helpful to sort out the drug resistance in *M. tuberculosis* as well as in mixed microbial flora. This improved system could further expand our understanding of *M. tuberculosis* pathogenicity, lead to identification of new therapeutic targets, and facilitate the development of highly effective anti-TB drugs and vaccines.

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