Mycobacteriophages: An important tool for the diagnosis of *Mycobacterium tuberculosis* (Review)

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Abstract. The prevention and control of tuberculosis (TB) on a global scale has become increasingly important with the emergence of multidrug-resistant TB. *Mycobacterium tuberculosis* phages have been identified as an important investigative tool. Phage genomes exhibit a significant level of diversity and mosaic genome architecture, however, they are simple structures, which are amenable to genetic manipulation. Based on these characteristics, the phages may be used to construct a shuttle plasmid, which is an indispensable tool in the investigation of TB. Furthermore, they may be used for rapid diagnosis and assessing drug susceptibility of TB, including phage amplified assessment and reporter phage technology. With an improved understanding of mycobacteriophages, further clarification of the pathogenesis of TB, and of the implications for its diagnosis and therapy, may be elucidated.

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1. Introduction

The emergence of multi-drug resistant tuberculosis (TB) has increased focus on the global prevention and control of TB. At present, TB control faces a number of challenges, including low sensitivity, poor specificity and treatment complications, long detection cycles of traditional diagnostic techniques

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and a decline in the immune function of traditional vaccines, including Bacillus Calmette-Guerin (1). Since 1947, when mycobacteriophages were first isolated and identified by Gardner *et al* (2), >3,680 types of mycobacteriophage have been isolated from different sources, of which >544 types of mycobacteriophages have undergone complete genome sequencing (3). As a member of the bacteriophage family, which are DNA viruses, mycobacteriophages are able to infect the host *Mycobacteria* specifically. L5 (4), D29 (5) and TM4 (6) are the mycobacteriophages, were the earliest to undergo genome sequencing and are the most widely used in the investigation of TB. Greater understanding of the structure and function of the mycobacteriophage genome has increased awareness of the importance of investigating the diagnosis and treatment of *Mycobacterium tuberculosis*.

2. Molecular tools of *Mycobacterium* investigation

In 1964, Tokunaga and Sellers (7) were the first to use the D29 phage to successfully induce outer DNA into *M. smegmatis*, which demonstrated the feasibility of mycobacteriophage DNA transfection. Subsequently, in 1970, I3 was successfully enriched with the use of *M. smegmatis* by Raj and Ramakrishnan (8), which again supported the viability of transduction. Since these early experiments, the rapid development of genetic engineering has led to the construction of a number of high-efficiency cloning and expression vectors. Recombinant DNA technology has also progressed, however, due to a lack of understanding of the mycobacteriophage genome, progress in recombinant DNA technology for the application of mycobacteria has been slow. Mycobacteria have a lipid-rich cell wall, which limits the ability of the exogenous DNA to pass through the cell wall and, therefore, foreign DNA are unable to be stably integrated and expressed in mycobacteria (9).

Jacobs *et al* (10) succeeded in creating a method of mycobacterial genome transfer in 1987, overcoming the difficulties in investigating mycobacterial genes. The plasmid DNA of *Escherichia coli* was inserted into the non-essential region of the TM4 genome, to successfully construct a recombinant shuttle plasmid vector (Fig. 1). The vector was a dual function shuttle plasmid vector, which was not only a plasmid replicated in the *E. coli*, but also a phage replicated within

the mycobacteria. Therefore, this overcame the deficiencies of traditional plasmid vectors, carrying a limited length of exogenous DNA fragments, and the insufficient capacity of bacterial transformation. The experimental results demonstrated that the recombinant shuttle vector was transfected into fast-growing *M. smegmatis,* however, this experiment was not successful in slow growing mycobacteria, including Bacillus Calmette-Guérin (BCG) strains and *M. tuberculosis*. Despite this, it demonstrated that recombinant shuttle plasmids may eventually be suitable for use to induce exogenous DNA into the BCG vaccine strains to develop a recombinant mycobacterial vaccine. Snapper *et al* (11) also constructed a shuttle plasmid successfully based on L1 and demonstrated the stable insertion and replication of exogenous DNA in *M. smegmatis*. Lee *et al* (12) achieved an effective and stable transformation using the mild site-specific integrated L5 mycobacteriophage. These findings demonstrated the building of an efficient integration vector by integrating the plasmid sequences into the mycobacterial genome, with effective integration of the TB mycobacterium and BCG to obtain stable recombinant DNA.

These previous studies demonstrated that the shuttle plasmid was of value for specific transduction (13), transposon transfer (14,15) and the introduction of diagnostic reporter genes (16,17). The development of this vector system promotes the genetic analysis *Mycobacterium* pathogens and the development of a recombinant vaccine.

3. TB diagnosis and drug sensitivity assessments based on mycobacteriophages

TB is the most important global public health problem at present. In 2010, there were 8.8 million incident cases of TB, 1.1 million deaths from TB among HIV-negative people, and an additional 0.35 million deaths from HIV-associated TB (18). Therefore, the control of the condition via rapid and accurate TB diagnosis is important. The demand for a simple, fast, safe, sensitive and accurate *M. tuberculosis* antibiotic susceptibility assessment has become increasingly urgent, as a result of the emergence and spread of multidrug-resistant TB and extensively drug-resistant tuberculosis (XDR-TB). In previous years, molecular techniques for the diagnosis of TB have been rapidly developed. The nucleic acid amplification method, involving nucleic acid probes, polymerase chain reaction, DNA sequencing, Gene Chip and Xpert MTB/RIF enables rapid diagnosis and assessment of resistance of *M. tuberculosis* (19). Although the majority of the techniques are fast with a high sensitivity, the requirement for specialized instruments and high costs significantly limited its dissemination and application in the majority of countries with a high burden of TB. In addition, there was a $1 billion gap in the funds of the World Health Organization for TB management and control in 2012, causing financial pressure in the diagnosis and treatment of TB (18). Assessments, which enable the rapid detection of mycobacteriophages have numerous advantages, including high speed, simplicity, specificity, security, no requirement for specialist equipment and lower costs, and they enable the quantitative detection of viable cells. Therefore, mycobacteriophages have become an ideal tool for TB diagnosis and assessment of drug susceptibility.

Phage amplification technology. The investigation of phage amplification technology can be traced back to 1965. A study by Sellers *et al* (20) observed the effects of anti-TB drugs on mycobacteriophages. The results of the experiments demonstrated that streptomycin (STR) was able to prevent the phages copying in *M. smegmatis*, whilst not affecting the phage replication of the progeny in resistant strains, or their subsequent release. Since this observation, other drugs, including clofazimine, colistin, rifampicin (RIF) and STR have also been assessed for their effects on the synthesis of D29 (21,22). In these studies, D29 was able to affect slow-growing pathogenic mycobacteria and the fast-growing environmental strains, and visible plaque formed in the fast-growing *M. smegmatis* bacteria following overnight incubation. The existence of viable bacteria can be determined by rapid detection of the release of progeny phages following infection of the mycobacterium target using this technique. These experiments laid the foundation for the subsequent development of phage amplification technology and its application in assessing anti-mycobacterial drug sensitivity.

The phage amplification technology, which is in current clinical use was first described by Wilson *et al* (23) in 1997, and further defined on the basis of further modifications by McNerney *et al* (24). Subsequently, Biotec Laboratories Ltd. (Ipswich, UK) developed corresponding commercial kits, *FASTPlaque*TB™ and *FASTPlaque*TB-*MDRi*™, or *FASTPlaque*TB-*RIF*™ (25,26), which were used for the rapid detection of *M. tuberculosis* and for the assessment of multi-drug resistance (Fig. 2). Firstly, D29 phages were introduced into *M. tuberculosis*, in which they reproduced. The phages, which did not enter the cell were killed by virucide agents, however, the phages that entered the viable *M. tuberculosis* were not affected. The phages lysed the bacteria *in vivo* following replication in the bacteria. Subsequently, the releasing phages infected and lysed *M. smegmatis* to form plaques. As this assessment is reliant on the presence or absence of plaques to determine the result, this method generally requires 1-2 days to produce results. As there is a proportional association between the quantity of plaques and the quantity of *M. tuberculosis* in the specimens, the content of *M. tuberculosis* in the sample can be calculated according to the number of plaques. As *M. tuberculosis* is cleaved during the experiment, the experiment has fewer safety concerns for the individuals involved.

The clinical effects of this assay have been evaluated in several countries, including Egypt (27), Pakistan (28), South Africa (29) and Spain (30). Kalantri *et al* (31) performed a meta-analysis of the detection of *M. tuberculosis* in clinical samples, based on phage amplification technology in 2005 by examining the literature from databases, including Medline, EMBASE (http://www.elsevier.com/online-tools/embase), Web of Science (http://wok.mimas.ac.uk/) and BIOSIS Previews (http://biosispreviews.isihost.com/). A total of 13 studies were included, which complied with designated standards. The specificity and sensitivity of these assays were between 0.83 and 1.00, and between 0.21 and 0.94, respectively, with sputum culture as a reference standard. The results revealed that the assay had a high specificity and a moderate/variable sensitivity, which required improvement. The predominant reasons for the lower sensitivity included anti-TB treatment prior to the experiment,
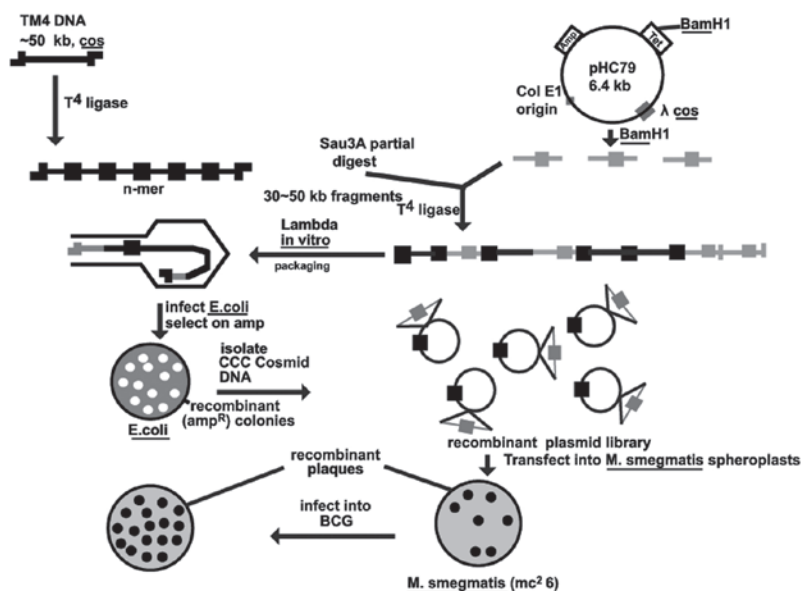
Figure 1. Diagram of the stages of construction of a shuttle plasmid. TM4 phage DNA is ligated and then partially digested with *Sau*3A to obtain fragments between 30 and 50 kb. These DNA fragments are then ligated to the cosmid pHC79, which has been cleaved with *Bam*HI. The ligation mixture is packed into the phage λ head *in vitro*. The resulting phages are transduced into *E. coli*. The clones containing recombinant pHC79::TM4 DNA molecules are selected on media containing ampicillin. The plasmid is isolated from the pool of clones and then transfected into *M. smegmatis* to select for the shuttle plasmid, TM4 phages containing pHC79. The shuttle plasmid is able to infect and replicate in BCG vaccine strains. BCG, Bacillus Calmette-Guerin.
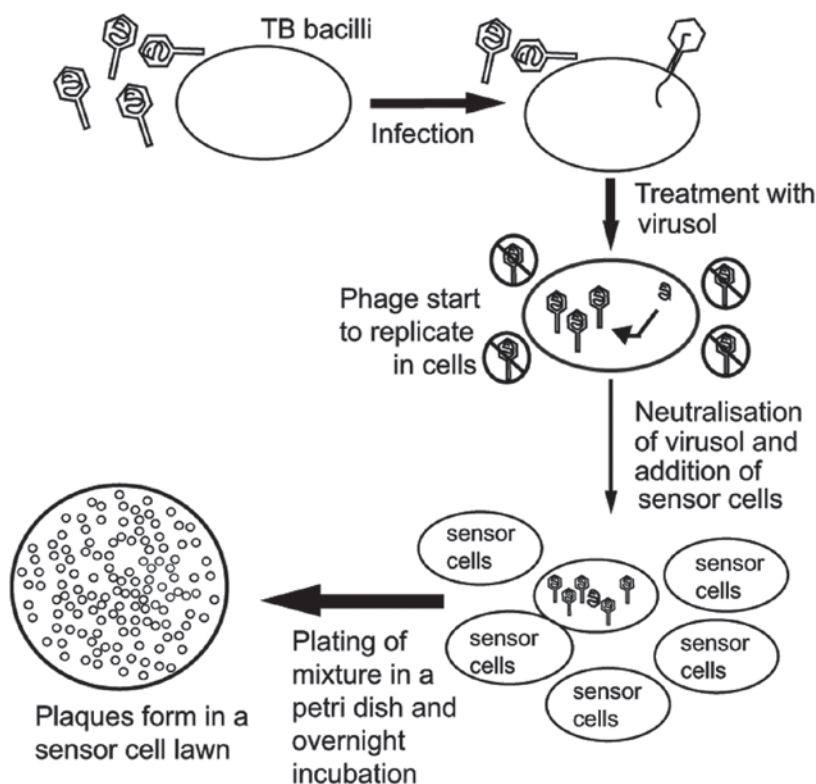

Figure 2. Diagrammatic representation of phage amplification technology. Mycobacteriophages infect TB bacilli in the sample. Following infection, the sample is treated with a potent virucide to destroy the mycobacteriophages that have not infected host cells. The remaining phages within the host organisms undergo a replication cycle, resulting in the release of the phages from the host cells. The released phages are allowed to infect a lawn of rapidly-growing, non-pathogenic mycobacteria (sensor cells). Clearing plaques indicate the presence of TB cells. TB, *tuberculosis*.

sample transportation, environmental conditions and the selection of detergents. Therefore, further investigations are required to improve the sensitivity of the phage-based assessment.

The Foundation for Innovative New Diagnostics (FIND) extensively evaluated the role of the *FASTPlaque* assessment technique in rifampin resistance in 2007 (32). The *FASTPlaque*

assay failed to achieve the desired objectives in two trial sites in South Africa. Therefore, FIND terminated the *FASTPlaque* assessment pilot program until a satisfactory improvement had been made (32). Therefore, although *FASTPlaque* assessment can be widely used for the rapid diagnosis of TB, however, further improvement of the optimization techniques is required.

Luciferase reporter phage. The fluorescent reporter phage is a rapid detection system for *M. tuberculosis* susceptibility and drug susceptibility based on recombinant DNA technology. The first generation of luciferase reporter phages (LRPs) were developed successfully by Jacobs *et al* in 1993 (16). These were constructed from the phAE39 plasmid shuttle, on the basis of TM4, and the firefly luciferase (*FFlux*) gene was inserted using a potent promoter of heat shock protein 60 (hsp60; Fig. 3). LRPs are able to transfer recombinant DNA into mycobacteria, including the *M. smegmatis* and *M. tuberculosis* BCG vaccine strains, In the presence of adenosine triphosphate and luciferin, *FFlux* is able to continuously express and generate an optical signal following mycobacterial infection. If there are at least 10^4/milliliter of *M. tuberculosis* in the sample, the relative light units can be detected within a few minutes following LRP infection of the live mycobacteria. This method reduced the reporting duration considerably compared with the traditional detection methods. LRPs based on L5 (33) and D29 (34) have been subsequently constructed, however, various defects remain. The mild L5 mycobacteriophage is unable to infect the *M. tuberculosis* complex, which limits its application in the drug resistance detection of clinical samples. The lytic characteristics of D29 and TM4 result in the loss of light output and reduced sensitivity. Since the characteristics of lytic phages may reduce light output, Kumar *et al* (35) constructed new LRPs using the mild Che12 bacteriophage to increase light output and improve the sensitivity of the assessment. Carriere *et al* (36) addressed the problem using a number of strategies, including changing the position of *FFlux* in the phage genome, isolating host-range mutant phages and inducing temperature-sensitive mutants of phages to screen more sensitive mutants compared with the first generation LRPs. Although the sensitivity of LRPs has improved, these LRPs can infect mycobacteria with the exception of *M. tuberculosis*, leading to misdiagnosis in clinical practice, therefore, it is necessary to improve the experimental program to confirm the presence of the *M. tuberculosis* complex. Considering these problems, Riska *et al* (37) added ǫ-nitro-α-acetylamino-β-hydroxy propiophenone to the substrate to selectively inhibit the *M. tuberculosis* complex bacteria, and combined the corrected program with the ordinary LRPs to accurately distinguish strains of the *M. tuberculosis* complex and non-TB mycobacteria, which improved the accuracy of the anti-TB drug susceptibility assessment.

As the phages only replicate in living cells, the limitations of the above methods include the ability to detect only viable cells in the sample. However, *M. tuberculosis* is dormant in the bodies of numerous patients with clinically latent infections (38), presenting a challenge in detecting dormant *M. tuberculosis*. Dusthackeer (39) used the *hsp60*,
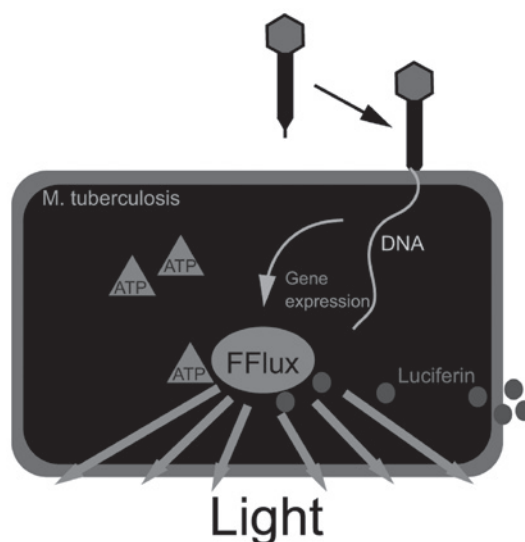

Figure 3. Schematic representation of the luciferase reporter phages. The mycobacteriophage, with a firefly luciferase gene (*fflux*) inserted within its genome, is used to infect M. *tuberculosis* within a sample. In addition to the enzyme, light output requires two substrates, luciferin and ATP. Luciferin is added exogenously and adsorbed rapidly by the M. *tuberculosis* cells. ATP is provided by the viable host cells. Therefore, following infection of the viable target cells, the *fflux* gene is expressed and light is produced. ATP, adenosine triphosphate.

isocitrate lyase and α crystal protein (α-crystallin) gene promoters to promote the gene expression of *FFlux*, and successfully detected the dormant *M. tuberculosis* bacteria. Dusthackeer *et al* (40) improved the experimental method further by detecting the sputum samples without the primary culture. It was suggested that this provided a better simulation of the natural state of dormant bacteria. The results of this study supported this hypothesis, which demonstrated the possibility of potential TB detection.

Banaiee *et al* (41) compared the assessment of the drug susceptibility of LRPs with the BACTEC 460 assay as a reference in clinical applications. The BACTEC 460 assay is a semi-automated phage-based antibiotic susceptibility assay. The results revealed that the diagnostic accuracy of LRPs reached 98.4%, and the drug detection accuracy rate was 100%. The sensitivity and specificity for the detection of RIF drug resistance were 100%, and for isoniazid (INH) were 100 and 97.7%, respectively. The duration required to perform an LRP trial was considerably reduced, just 2 days, compared with the BACTEC 460 assessment, which required 9 days. In addition, its economic cost is low, at $0.40 for each strain. This semi-automated LRP assessment technique is ideal for laboratories with limited funds, enabling assessments in economically underdeveloped countries experiencing a high burden of TB. Minion and Pai (42) performed a meta-analysis of the phage-based assessments of RIF resistance prior to 2009, a total of 31 studies were included in a sample of 3,085 studies, and the phage amplified biological assessment and LRP assessment were compared. The results revealed that the sensitivity and specificity of the LRPs were 99.3 and 98.6%, marginally higher than the phage amplified biologically assessment at 98.5 and 97.5%. However, a similar investigation with a larger LRP sample size is required.
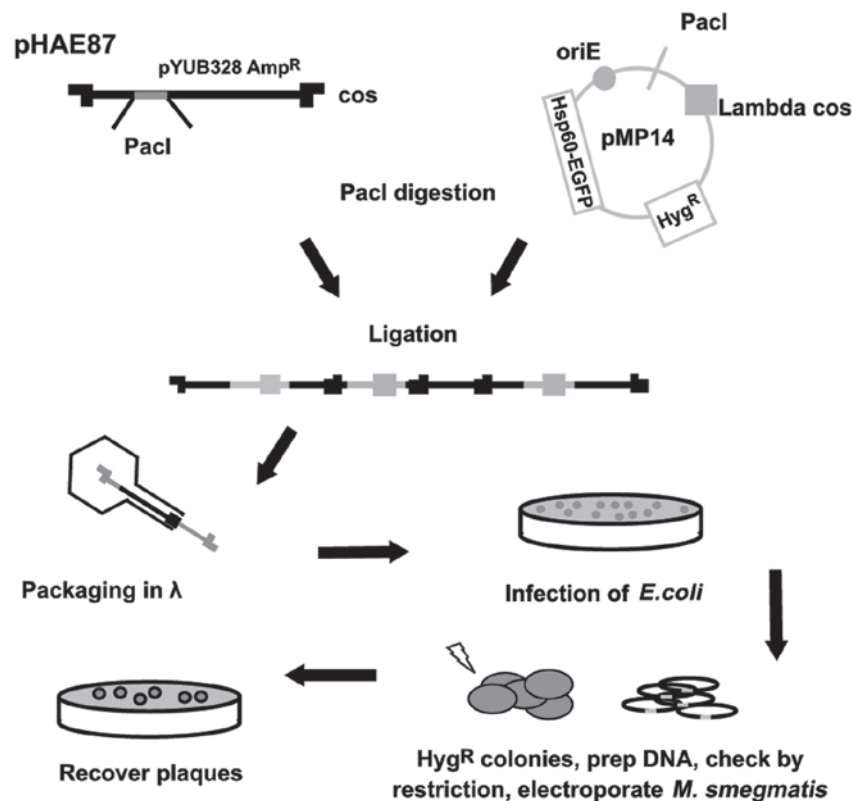
Figure 4. Fluoromycobacteriophage construction. Schematic representation of phAE87::*hsp60-EGFP* construction. The phAE87 huttle plasmid is a conditionally replicating derivative of phage TM4, in which the cosmid moiety is flanked by *Pac*I restriction sites. A plasmid derivative of pYUB854, containing the EGFP gene (pMP14), is used to replace the cosmid in phAE87. This is followed by λ packaging and recovery in *E. coli*. EGFP, enhanced green fluorescent protein; Hyg[R], hygromycin-resistance marker; Amp[R], ampicillin-resistance marker.

Fluoromycobacteriophages. Fluoromycobacteriophages, a novel phage, were identified in 2009, and differ from the previously reported LRPs. Piuri *et al* (17) constructedthe fluoromycobacteriophages phAE87::*hsp60-EGFP* and phAE87::*hsp60-ZsYellow* (Fig. 4). Green fluorescent protein *(GFP)* or the *ZsYellow* fluorescent markers were introduced into *M. tuberculosis*, to detect the drug susceptibility using fluorescence microscopy or flow cytometry within 24 h. The technique has several advantages compared with the LRPs, as no substrate is required, <100/ml *M. tuberculosis* can be identified, drug-resistant strains may be detected in the mixed population and the biosecurity of the samples is enhanced by polyformalin-fixed processing. Rondón *et al* (43) also designed an enhanced GRP (EGFP) phage, phAE87:: *hsp60-EGFP,* containing *EGFP* on the basis of TM4. This technique was used to detect drug resistance of *M. tuberculosis* strains to INH, RIF and STR, and the results revealed that the sensitivity of this technique to all antibiotics was 94%, and the specificities of INH, RIF and STR were 90, 93 and 95%, respectively, compared with the resazurin microplate technique. The results of the resazurin microplate assay also exhbited 94% sensitivity for INH and RIF, whereas sensitivity for STR was higher at 98%. The reporting time-period of this technique was 2-3 days and the costs were ~$2. Although *EGFP* phage technology for rapid screening of combined drug resistance is of potential economic value, it requires further simplification to suit clinical requirements as a rapid and economic way to detect multidrug-resistant or extensively drug-resistant strains of TB in resource-poor settings with minimal infrastructure, and improve sensitivity.

One problem of fluoromycobacteriophages is that, as a potent mycobacteriophage, TM4 initially infects bacteria, and then cleaves it, terminating the expression of EGFP. Therefore, the sensitivity of fluoromycobacteriophages is reduced as the duration of EGFP expression is shortened (43). To address this problem using bacteriophage recombineering of electroporated DNA, da Silva *et al* (44) inserted a P$_{hsp60}$-*egfp* cassette into the D29 mycobacteriophage genome to construct a novel reporter phage. Based on the this novel reporter phage, an attempt was made to construct a lysis-defective mutant by deleting the lysA gene, however, it was not possible to purify the mutant. Despite this, the attempt provided a novel strategy for the development of a more sensitive reporter phage.

Another problem of fluoromycobacteriophages is that the adsorption of TM4 is relatively inefficient. However, mutants can be isolated with enhanced adsorption, which may provide a strategy for improving the efficiency of recovery. Piuri *et al* (45) constructed a plasmid expressing the major capsid protein gene (gp9) of TM4, and containing Strep-tag II (STAG II). Particles with capsids composed of wild-type and STAG-tagged subunit mixtures were able to grow to high titers, exhibited good infectivity and were suitable for used to isolate phage-bacterium complexes. Reporter phage technology based on the fluorescent protein emitting principle requires further evaluation of its clinical effects.

4. Conclusion

Since mycobacteriophages were identified 50 years ago, >2,439 types of mycobacteriophages have been isolated and the genome sequences of >363 types of mycobacteriophages have been completed. Mycobacteriophage genomes have several features, including diversity and mosaicism, a simple structure and amenability to genetic manipulation. Based on these characteristics, a shuttle plasmid was constructed for TB investigation using recombinant DNA technology. With improvements in genomics, shuttle plasmids have also been used to build different luciferase reporter phages and fluoromycobacteriophages, which have contributed to the investigation of mycobacteria and TB. Following several years of limited studies, phage therapy is again an active area of investigation, particularly in bacteriophage lyase. As investigation into mycobacterial phages progresses, improvements in the current understanding of its role in TB, and particularly its diagnosis and treatment, is expected.

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