Published in final edited form as:

Tuberculosis (Edinb). 2008 November ; 88(6): 616–623. doi:10.1016/j.tube.2008.02.007.

Characterization of temperate phage Che12 and construction of a new tool for diagnosis of tuberculosis

Vanaja Kumar¹, Prabakaran Loganathan¹, Gomathi Sivaramakrishnan¹, Jordan Kriakov², Azger Dusthakeer¹, Balaji Subramanyam¹, John Chan², William R. Jacobs Jr², and Narayanan Paranj Rama¹

¹Tuberculosis Research Centre, Chennai, India

²Albert Einstein College of Medicine (AECOM), New York, 10461, USA.

SUMMARY

A temperate phage, Che12, able to infect *Mycobacterium tuberculosis*, was isolated from soil samples taken from tuberculosis sanatorium area in Chennai, India. The plaque morphology of this phage showed varying grades of turbidity on lawns of *M. tuberculosis*. The temperate nature of Che12 was established by super infection immunity. Phage integration into the host genomic DNA was confirmed by Southern hybridization using Che12 DNA as a probe. PCR amplification and sequencing of a part of the integrated phage genome in a *M. tuberculosis* lysogen also confirmed the temperate nature of Che12. The morphology of the phage particles was observed by electron microscopy, revealing similarities to other mycobacteriophages like L5, D29 and TM4. A luciferase reporter phage, phAETRC16, was constructed by cloning firefly luciferase gene into Che12. Infection of viable *M. tuberculosis* cells by phAETRC16 resulted in expression of luciferase leading to sustained light output. Che12, a true temperate phage infecting *M. tuberculosis*, is thus ideally suited for developing a diagnostic tool facilitating rapid diagnosis of *M. tuberculosis*.

Keywords

M. tuberculosis; temperate; luciferase reporter phage

INTRODUCTION

Early studies evaluated the morphology of mycobacteriophages and established their use as diagnostic as well as epidemiological tools for detecting and typing pathogenic mycobacteria. ^{1–3} The role of mycobacteriophages as valuable tools in the development of mycobacterial genetics has been reviewed. ⁴

Most recent efforts have been focused on particular phages because of their specific attributes. I3, a generalized transducing phage, ^{5,6} D29 and TM4, the lytic phages infecting *M. tuberculosis* and L5, a temperate phage originally infecting mycobacteria other than *M. tuberculosis* complex and found to infect *M. bovis* BCG under specific conditions ⁷ have been

Corresponding author and address Vanaja Kumar, Deputy Director, Tuberculosis Research Centre (Indian Council of Medical Research), Mayor V.R. Ramanathan Road, Chetput, Chennai, 600 031. India. Phone: +91-44-2836 9659, E-mail: E-mail: vanaja_kumar@hotmail.com Fax: 91-44-2836 2525.

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extensively used to facilitate genetic analysis of mycobacteria. The use of mycobacteriophages as vectors was initiated by the construction of recombinant shuttle plasmids based on the lytic mycobacteriophage TM4.⁸ Shuttle plasmids are chimeric DNA molecules consisting of a cosmid integrated into the nonessential regions of a phage genome. With this technology, a number of plasmids from TM4, D29 and L5 have been constructed and used in the development of luciferase reporter phages (LRP).⁸ Using these reporter phages to deliver the firefly luciferase (*Fflux*) gene into mycobacteria it has been possible to develop highly sensitive tests for detection of drug resistance in clinical isolates of *M. tuberculosis* and *M. avium* and diagnosis of *M. tuberculosis*.^{8–11} The luciferase reporter phage assay was highly specific but required 10⁴ organisms per ml to give a detectable level of light output expressed as relative light units (RLU) in a luminometer.¹²

Luciferase reporter phages developed from temperate phage L5 showed increased light output from *M. smegmatis* compared to lytic phage constructs¹³ suggesting that a similar construct from temperate phage infecting *M. tuberculosis* would bring about a sustained light output leading to better sensitivity of LRP assay. An attempt to isolate new phages from soil samples of Chennai, India was initiated in search of such a phage so as to develop a diagnostic LRP assay. Of the many phages isolated, phage Che12 isolated at Tuberculosis Research Centre, Chennai was found to be a temperate phage capable of infecting and lysogenising *M. tuberculosis*. We describe here the isolation and characterization of the phage Che12. A luciferase reporter phage (LRP) was developed from Che12 and its ability to detect *M. tuberculosis* was evaluated.

MATERIALS AND METHODS

Specimens

Moist soil samples from near cowsheds, hencoops and TB clinics in the Chennai area of India were collected in sterile containers.

Isolation of phages

To about 10g of the soil sample, 10 to 15 mL of MP buffer (Tris-HCl pH 7.5 50mM, NaCl 150mM, MgSO₄ 10mM, CaCl₂ 2mM) and 1 ml of either *M. smegmatis* mc²155 or *M. tuberculosis* H37Rv suspension equivalent to McFarlands standard # 2 was added and incubated at 37°C overnight. Supernatant was filtered through 0.45µ membrane. About 2 ml of the filtrate and 1 ml suspension of the host organism were mixed and incubated at 37°C for 30 minutes and 3 hours for *M. smegmatis* mc²155 and *M. tuberculosis* respectively. About 1 ml of the cell - phage mixture was mixed with 5 ml of soft agar and poured on Middlebrook's 7H9 plates. The plates were incubated at 37°C for 2 to 3 days for *M. smegmatis* mc²155 and up to 2 weeks for *M. tuberculosis*. Typical discreet plaque was purified for at least 3 generations and high titer lysates were prepared.

Host Range

On lawns of pathogenic species of mycobacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, 3 µl of neat and 10 fold dilutions of phage lysate were spotted and incubated at 37°C until the host cells form a continuous sheet of growth. Wherever clearance was seen in neat and in at least in 10⁻¹ dilution, the phage is considered to be infecting that host.

Isolation of lysogens

M. smegmatis mc²155 colonies growing within the turbid plaque of Che12 were isolated and purified on 7H9 agar plates for 3 generations to get lysogens of *M. smegmatis* containing Che12

genome. Out of these, three lysogens were used for Southern hybridization experiment while one of them was used for all other characterization experiments. However, developing lysogens of *M. tuberculosis* was difficult due to their extremely slow growth rate. A recombinant of Che12 phage was constructed with a hygromycin cassette and used for this purpose. Briefly, shuttle phasmid pAETRC10 was constructed as described previously⁸ and by replacing pYUB328 with vector carrying the hygromycin resistance marker, the recombinant Che12 construct was developed. By infecting *M. tuberculosis* and plating a portion of the phage-cell mixture on hygromycin (150 µg/ml) containing 7H11 medium, the *M. tuberculosis* lysogen of Che12 bearing the hygromycin resistance cassette was isolated.

Characterization of phage

1. Physiological characterization

Plaque morphology: Plates showing individual plaques were chosen to study the plaque morphology. The parameters such as clear/ turbid nature of the plaques and pleiomorphism were noted.

Phage Morphology: Sample preparation: Thirty milliliters of high titre phage lysate in MP buffer was ultra centrifuged in a 15% glycerol gradient, for 18 – 20 hrs at 22,000 rpm. The phage pellet was suspended in 0.5 ml of MP buffer and filtered through a 0.45 µ membrane filter.

For electron microscopic observation 10 µl of Che12 phage lysate in MP buffer, was placed in a 300-mesh grid. The excess was blotted using # 50 filter paper after standing for 2 minutes. About 10 µl of 1% phosphotungstic acid (pH 7.0) was added and the sample was observed under electron microscope (FEI Morgagni 268 with an AMT digital camera system) after blotting the excess. The accelerating voltage used was 80 kilovolts and phage morphology was observed under 140000x magnification.

Determination of the time of progeny release: A suspension of *M. smegmatis* mc²155 in Middlebrook 7H9 broth was prepared and adjusted to a concentration of 0.8×10^9 colony forming units/ml. Six millilitres of this suspension were mixed with an equal volume of phage Che12 (titre 1×10^{11} pfu/ml) and incubated at 37°C. At time intervals of 30 minutes up to 2 hours and at intervals of 15 minutes from then on up to 3 hours, 1 ml aliquots of the phage cell mixture were centrifuged at 12000 rpm for 5 minutes. The supernatant was serially diluted, mixed with an equal volume of *M. smegmatis* mc²155, plated on 7H9 agar medium and incubated at 37°C. The number of plaques was counted to determine the time taken for progeny release.

Super infection immunity: Che12 lysogen of *M. smegmatis* mc²155 was plated on 7H9 agar. About 3 µl of ten fold dilutions of D29, L5, Bxz2 and Che12 were spotted onto the lawn and incubated at 37°C for 48hrs. The clearing of lawn corresponding to the spots, if any, was noted.

Induction of the phage from lysogen: Suspension of *M. smegmatis* mc²155 lysogen of Che12 was made in Middlebrook 7H9 broth. About 500 µl of this suspension was subjected to the action of ultraviolet light from a 30 W (PHILIPS) tube for half an hour, mixed with 500 µl of *M. smegmatis* mc²155 suspension and 10 ml of molten 7H9 agar and poured on to the plate. Similarly after exposure to a higher temperature at 55°C for 30 minutes, about 500 µl was mixed with equal volume of *M. smegmatis* mc²155 suspension and 10 ml of molten 7H9 agar and poured on to the plate.

2. Molecular characterization

Lysogenicity in *M. smegmatis* mc²155: *Southern Hybridisation:* DNA isolated from Che12 phage, *M. smegmatis* mc²155 and three *M. smegmatis* mc²155 lysogens of Che12 by phenol chloroform extraction were digested with *Cla*I restriction enzyme for 30 minutes, run on 1% agarose gel, transferred to nylon membrane and hybridized with [³²P] labeled *Cla*I digested Che12 DNA as probe.

Lysogenicity in *M. tuberculosis*: Lysogens of Che12 in *M. tuberculosis* were extremely tiny in size and very slow to grow that sufficient DNA material could not be accomplished for performing Southern hybridization experiment. Instead, the following confirmatory experiment was done for demonstration of the integration of the phage into the genome of *M. tuberculosis*. Primer sets for two regions of length of 1.37 Kb between 19936 bp and 21309bp (region A) and of 1.19 Kb size between 34901 bp to 36096 (region B) flanking the *attP* site were designed using the sequence analysis details of Che12 phage genome (Accession number: DQ398043).

Primer set for region A:

Upstream : 5'GGACATCGAGGACAACTCTG 3'

Down stream: 5'ACCGTCCTTGATCCATCCAT 3'

Primer set for region B:

Upstream : 5'GTCGTCCTGCGACCAGTTCT 3'

Down stream: 5'GGCAAGGCATCAAGCCTCAC3'

Using *M. tuberculosis* integrating vector-transformed cell DNA as template, amplification of the two sites was done simultaneously under the following conditions. Initial denaturation was done for 5 minutes at 94°C followed by 35 cycles of 94°C for 40 seconds, 52°C for 40 seconds, 68°C for 90 seconds and a final extension of 7 minutes at 68°C.

Construction of LRP: Construction of LRP from Che12 was done in two stages. Initially, shuttle phasmids were developed using cosmid cloning as described earlier.⁸ Cosmid vector pYUB 328 was introduced in to the non-essential region of Che12. In the second stage, the vector was excised using restriction enzyme *Not*I and pYUB556 bearing the luciferase cassette was substituted in its place.¹⁰

Measurement of light production kinetics in *M. smegmatis* mc²155 and *M. tuberculosis*: Equal volume of *M. smegmatis* mc²155 or *M. tuberculosis* suspension in Middlebrook 7H9 broth (0.8×10^9 cfu/ ml) and high titer phage (1×10^{10} pfu/ ml) of Che12 LRP construct and phAE129¹⁴ were incubated at 37°C. At different time intervals, 100 µl of 0.3 mM D-luciferin (Sigma) in 0.05 M sodium citrate buffer pH 4.5 was added to 100 µl of the cell-phage mixture and RLU measured at 10 seconds integration time using the luminometer (Monolight 2010). Reproducibility of the time kinetics was reassessed with two standard strains viz. *M. tuberculosis* H₃₇Rv and Erdman strains and eight clinical isolates of *M. tuberculosis*.

RESULTS

Isolation of Phages

A temperate mycobacteriophage was isolated from soil sample near Tambaram Sanatorium, Chennai, India, using *M. tuberculosis* H₃₇Rv as the host organism. This phage named Che12, was found to form turbid plaques on *M. tuberculosis* bacterial lawns and was also capable of infecting *M. smegmatis* mc²155.

Plaque morphology

Plaques of Che12 appeared clear on *M. smegmatis* mc²155 lawn after overnight incubation. They turned turbid with the appearance of alternate rings on further incubation. After about 5 days, the plaques presented a characteristic folded appearance probably due to overlapping of the neighboring plaques of different grades of turbidity (Figure 1).

Phage morphology by Electron Microscopy

When viewed under a transmission electron microscope, Che12 showed similar morphology as that of TM4, L5 and D29. The phage head was isometric and icosahedral and the tail was long and flexible. This morphology is shared by a majority of the mycobacteriophages so far studied (Figure 2).

Host Range

Che12 exhibited a wide host range infecting almost multiple mycobacterial species such as, *Mycobacterium tuberculosis* H₃₇Rv, *Mycobacterium vaccae*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium microti*, *Mycobacterium diernhoferi*, *Mycobacterium. flavescence*, *Mycobacterium chitae*, *Mycobacterium gordonae*, *Mycobacterium aurum*, *Mycobacterium parafortuitum*, *Mycobacterium phlei*, and *Mycobacterium bovis*. Species such as *Mycobacterium xenopi*, *Mycobacterium simiae* or *Mycobacterium terrae* and other bacterial strains were not infected under the conditions tested.

Time of progeny release

The number of phages released following phage infection was measured at different time intervals. There was an initial two log decrease in the number of free phages up to 30 minutes probably corresponding to the adsorption of phages to the host cells. A steady increase in plaque forming units was observed from 30 minutes onwards reaching the peak at 1 hour and maintaining at almost the same level up to 3 hours indicating that maximum number of virions gets released at 1 hour and after.

Super infection immunity

The neat lysate of Che12 and tenfold dilution up to 1 in 10,000 did not show any plaque formation while D29, L5 and BxZ2 showed clearance on the lawn of *M. smegmatis* lysogen.

Induction of Che12 from the lysogen

A suspension of *M. smegmatis* mc²155 lysogen of Che12 in 7H9, when induced by exposure to UV or temperature at 55°C for 30 minutes and plated with wild type *M. smegmatis* mc²155, was found to release phages.

Lysogenicity in *M. smegmatis*

Southern Hybridisation—Hybridisation of the *Cla*I digest of three *M. smegmatis* mc²155 lysogens of Che12 DNA with *Cla*I digested, ³²P labeled Che12 DNA as probe, showed bands identical to that of the wild type phage (positive control). No such bands were seen hybridizing in the negative control using wild type *M. smegmatis* mc²155 DNA. This demonstrates the integration of the phage into the genome of the host (Figure 3).

Lysogenicity in *M. tuberculosis*

Gel electrophoresis of the amplified products revealed the presence of two bands of size 1.3 Kb and 1.19 Kb, which corresponded to the size of regions A and B respectively in the wild type phage. The amplified products of region A, when sequenced revealed 99% homology between the wild type Che12 genome and the genome of the *M. tuberculosis* lysogen. A

homology of 79% was observed for region B. The high homology thus exhibited by the genomes of the phage and the lysogen for the two regions flanking the *attP* is strongly indicative of the integration of the phage into the host (Figure 4).

Construction of LRP

Our attempt to develop LRP resulted in several constructs. Ten of these were tested with *M. smegmatis* and *M. bovis* BCG and the construct phAETRC16 showing maximum light output was chosen for further testing.

Light production kinetics of phAETRC16

When *M. smegmatis* mc²155 was infected with phAETRC16, a steady increase was observed in the light output up to 72 hours. The increase in the RLU was by 3 logs at 72 hours. Light production in the control phage phAE129 was maximum at 4 hours after which there was a gradual decrease with fluctuating bouts of signal output (Figure 5A).

Light production in a clinical isolate of *M. tuberculosis* infected with phAETRC16 showed an increase by 4 logs in 7 days. The control phage phAE129 showed the maximum light output at 8 hours after which there was a steady decline (Figure 5B).

The standard strains of *M. tuberculosis* when tested with phAETRC16 showed an increase from 2 logs to 5 logs in 5 days. All the 8 clinical isolates showed an increase from 2 fold to 5 fold by the 5th day (Figure 6). Wherever measured, the increase was maintained even on the 7th day. Two isolates showed a further increase by 4 fold.

DISCUSSION

Many of the already known mycobacteriophages have very similar morphology, independent of the genetic relatedness as assessed by DNA sequence similarity. However, plaque morphology of the temperate mycobacteriophages varies considerably. Bxz2 forms turbid plaques similar to those of L5 and Bxb1, from which stable lysogens can be recovered. The phage Cjw1 forms hazy plaques at 37°C but turbid plaques at 42°C.¹⁵ The temperate mycobacteriophage Bxb1 exhibits an unusual feature of forming halos surrounding the plaques that can expand to cover a large area of uninfected cells.¹⁶ Che12 is unique in forming clear plaques after overnight incubation on *M. smegmatis* mc²155 lawn, becoming turbid on further incubation. The individual plaques show different grades of turbidity resulting in a unique pattern of folding when the plaques are formed very close to each other.

Phage particles of Che12 showed isometric icosahedral heads and long flexible tail similar to that of the well characterized phages such as D29, L5, TM4 and other phages such as Bxb1, Bxz2, Che8, Omega, and Che9d. Bxz1 has a large icosahedral head and short contractile tail. Che9c and Corndog showed a different morphology with flexible tails but unusual prolate heads.¹⁵

Mycobacteriophage L5 as well as other very closely related phages including L1 and D29 exhibit a broad host range infecting mycobacteria including *M. tuberculosis* complex. Mycobacteriophage DS6A has a very narrow host range as it infects only *M. tuberculosis* complex. Because of its narrow host range and strain preference it has very limited use in diagnostic mycobacteriology. Phages with wide host range are ideal for developing molecular tools. Che12 has wide host range infecting more than 12 mycobacterial species including those of clinical importance and it does not infect other bacterial strains tested making it an ideal molecular tool.

Molecular aspects of mycobacteriophages have been extensively studied in the last two decades to develop tools to study the genetics of mycobacteria. L5, D29 and TM4 phages have been widely used to facilitate the genetic analysis of mycobacteria.¹⁷ The use of mycobacteriophages as vectors was initiated by the construction of recombinant shuttle plasmids based on the lytic mycobacteriophage TM4.⁸ Subsequently, luciferase reporter phages were constructed using D29¹⁰ and TM4.⁹ As a diagnostic tool, these first generation luciferase reporter phages were highly specific but lacked in sensitivity. The reporter phages developed later from TM4 showed better light production but still falling short in sensitivity to qualify as diagnostic tool. LRP developed from L5 was highly sensitive resulting in increased light output with *M. smegmatis* compared to those of lytic phages.¹³ L5, originally infecting mycobacteria other than *M. tuberculosis* complex was found to infect *M. bovis* BCG under specific conditions requiring a high concentration of Ca^{2+} .⁷ However, it is not capable of infecting *M. tuberculosis*.

Thus isolation of temperate mycobacteriophage infecting *M. tuberculosis* would provide an ideal candidate for molecular studies as well as for construction of sensitive luciferase reporter phages exhibiting sustained production of light. With this aim, the present attempt was made to isolate mycobacteriophages from soil, at locations frequented by tuberculosis patients. Che12 thus isolated in the present study had been found to infect both fast and slow growing species of mycobacteria. Lysogenicity of Che12 in *M. smegmatis* was established by super infection immunity and Southern hybridization. Confirmation of lysogenicity in *M. tuberculosis* necessitated the isolation of the lysogen, which was unsuccessful using the wild type phage by standard procedures. Alternatively, a recombinant construct of the phage carrying hygromycin resistance cassette, facilitated the isolation of the lysogen. Presence of the integrated phage genome in the *M. tuberculosis* lysogen was demonstrated by amplification and sequence analysis to establish lysogenicity. Regions A and B of the phage genome flanking the *attP* site were amplified using the DNA of *M. tuberculosis* lysogen as template and sequence analysis of the amplified products showed a homology of 99% and 79% respectively with that of the wild type Che12 genome. In an earlier report, lysogeny in *Lactobacillus casei* has been established by the demonstration of the presence of an antibiotic marker through sequence analysis by Shimizu-Kadota and Sakurai in 1982.¹⁸ Alternatively, in 2004, Li et al.,¹⁹ demonstrated lysogeny by phage induction and Southern blotting with Micromonospora phage PhiHAU8.

In silico analysis of the entire genome of Che12 identified genes coding for enzymes required to lysogenise with stoperators present at 16 sites exhibiting similarity of *attP* of Che12 to *attB* of *M. tuberculosis* strengthening the fact that Che12 can successfully lysogenise *M. tuberculosis*.²⁰

Che12, L5 and D29 constitute a closely linked family of homoimmune mycobacteriophages. These phages can be potentially used for genetic engineering of mycobacteria and diagnosis of mycobacterial infection. The effectiveness of such phage-based systems depends on the efficiency with which they infect and grow within target cells.²¹ Che12 described in the present study is the first true temperate phage infecting *M. tuberculosis* and the Che12 based LRP construct phAETRC16 shows a steady increase in light output while infecting clinical isolates. The sustained production of light, steadily increasing even beyond seven days provides flexibility for designing different assay formats suitable for different laboratory situations.

Introduction, stabilization and expression of foreign antigen genes are vital steps required for the construction of live recombinant vaccines. Temperate phages offer a rich collection of tools for site-specific integration and vaccine construction. Integrative vectors using the integrase and the *attP* of L5 have been developed establishing the role of mycobacterial integration host

factors, essential for site-specific recombination²² and also find application in development of recombinant strains.

Establishment of a genetic system using a non-antibiotic resistance genetic marker is ideal for mycobacteria due to the innate resistance of bacteria to antibiotics. Temperate phages have this potential as observed by the use of L5 gp71 selection for the construction of recombinant bacterial vaccines.^{23–24}

Description of both temperate and generalized transducing phages provides an opportunity to investigate the genetics of both phage as well as its host.²² The approach using genomics of temperate phages of *Staphylococcus aureus* has been employed to identify phage ORFs with antimicrobial activity and their cognate bacterial targets for subsequent drug discovery.²⁵ In 1998, Yoon et al.,²⁶ used temperate phages for Cre/loxP-mediated excision and amplification of large segments of the *E. coli* genome. Shimizu-Kadota (2001)²⁷, reported the stability of the final integrant, in the bacterial chromosome for up to 50 generations in *Lactobacilli* signifying the robustness of the temperate phages.

CONCLUSION

Che12 is a unique phage being the first temperate mycobacteriophage isolated and characterized, capable of infecting and lysogenising *M. tuberculosis*. Its temperate nature makes it an ideal candidate for developing diagnostic and molecular tools facilitating rapid diagnosis and better understanding of *M. tuberculosis*. In addition, construction of a cosmid library of the *M. tuberculosis* genome using Che12 could find application as knock-in phages that could be of immense value for the study of gene related functions of the host.

Acknowledgement

VK, LP, NSG and AD each acknowledge support from the AIDS International Training and Research Program (NIH D43-TW01403) of the Albert Einstein College of Medicine (Program Director: Vinayaka Prasad).

SB acknowledges the support by the World Health Organization with funds from the United States Agency for International Development through South East Asia Regional Office, New Delhi, India.

Authors thank Deborah Jacobs-Sera, Marisa Pedulla, Graham Hatfull and Roger Hendrix of Bacteriophage Institute, University of Pittsburg, Pennsylvania for help with electron micrograph, providing the sequence details of Che12 and for critically going through the manuscript, respectively.

Authors thank Santhakumar Thirupapuliur of Albert Einstein College of Medicine, Yeshiva University, New York for help in performing Southern hybridization experiments.

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Figure 1.

Plaques of Che12 appear clear initially turning turbid in about 5 days exhibiting different grades of turbidity. On Middlebrook 7H9 agar plates the plaques formed very close to each other resulted in a special folded appearance due to overlapping of neighboring plaques with varied grades of turbidity.

Figure 2.
Electron micrograph showing isometric, icosahedral head with long flexible tail. (Print magnification $\times 1,35,000$)

Figure 3. Southern hybridisation of the *Cla*I digest of Che12 lysogens of *M. smegmatis* mc²155 DNA (lanes 3,4,5) using *Cla*I digested, ³²P labeled Che12 DNA as probe, revealed identical band pattern as the wild type phage Che12 (positive control - lane1). The negative control using wild type *M. smegmatis* (lane 2) did not show any band hybridizing with the probe.

Figure 4. Gel electrophoresis of the PCR products of the regions A (1.3 kb) and B (1.19 kb) from *M.tuberculosis* lysogen in lanes 2 and 3 respectively, Lane 1: 1 kb ladder.

Figure 5.

Comparison of light production kinetics of phAE129 and phAETRC16 in *M. smegmatis* mc²155 [A] and *M. tuberculosis* [B]. The relative light unit (RLU) was 1 log higher than that of phAETRC16 at 4 hours. Steady increase in light out put thereafter resulted in a difference of 3 logs as compared to phAE129 at 72 hours [A]. RLU production by phAETRC16 in *M. tuberculosis* was seen only at 24 hrs. It increased steadily up to 7 days producing a light out put of 7 digits RLU. The light production of phAE129 started as early as 4 hrs showing 5 digits RLU up to 48 hours [B].

Figure 6.

Light production kinetics of phAETRC16 in clinical isolates and standard strains of *M. tuberculosis*. Clinical isolates of *M. tuberculosis* (1–8), *M. tuberculosis* Erdman (9) and *M. tuberculosis* H37Rv (10) produced minimal light production at 6 hrs increasing steadily resulting in RLU of 6 digits at 5 days and beyond.