**Isolation and characterization of bacteriophages from India,
with lytic activity against *Mycobacterium tuberculosis*.**

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Complete List of Authors:	Bajpai, Urmil; Acharya Narendra Dev College Mehta , Abhishek; Acharya Narendra Dev college Eniyan, Kandasamy; Acharya Narendra Dev college Sinha, Avni; Acharya Narendra Dev college Ray, Ankita; Acharya Narendra Dev college Virdi, Simran; Acharya Narendra Dev college Ahmad, Shazeb; Acharya Narendra Dev college Shah, Aridni; Acharya Narendra Dev college Arora, Deepanksha; Acharya Narendra Dev college Marwaha, Devyani; Acharya Narendra Dev College Chauhan , Gunjan; Acharya Narendra Dev college Saraswat , Prarthna; Acharya Narendra Dev college Bathla , Punita ; Acharya Narendra Dev college Singh , Ruchi; Acharya Narendra Dev college
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2 **lytic activity against *Mycobacterium tuberculosis*.**

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4 *Urmi Bajpai (Ph.D.), Abhishek Kumar Mehta (Ph.D.), Kandasamy Eniyan (M.Sc.), Avni*
5 *Sinha (M.Sc.), Ankita Ray (M.Sc.), Simran Virdi (B.Sc.), Shazeab Ahmad (B.Sc.), Aridni*
6 *Shah (B.Sc.), Deepanksha Arora (B.Sc.), Devyani Marwaha (B.Sc.), Gunjan Chauhan*
7 *(B.Sc.), Prarthna Saraswat (B.Sc.), Punita Bathla (B.Sc.), Ruchi Singh (B.Sc.).*

8
9 *Department of Biomedical Science, Acharya Narendra Dev College (University of*
10 *Delhi) Govindpuri, Kalkaji, New Delhi-110019, India.*

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12 **Running Head:** Lytic bacteriophages capable of infecting *M. tuberculosis*

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16

17 **Correspondence**

18 Dr. Urmi Bajpai (Associate Professor)
19 Department of Biomedical Science,
20 Acharya Narendra Dev College (University of Delhi)
21 Govindpuri, Kalkaji, New Delhi-110019, India.

22 Contact Number: +91-9811299719

23 Email id: urmibajpai@andc.du.ac.in

24 Fax Number: +91-11-26412547

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26 **Abbreviations**

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28 PDRP, Phage Discovery Research Project; TMP, tape measure protein;

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38 ABSTRACT

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40 Bacteriophages are being considered as a promising natural resource for the
41 development of alternative strategies against mycobacterial diseases, especially in the
42 context of the wide spread occurrence of drug-resistance amongst the clinical isolates
43 of *M. tuberculosis*. However, there isn't much information documented on
44 mycobacteriophages from India. Here, we report the isolation of 17
45 mycobacteriophages using *M. smegmatis* as the bacterial host where 9 phages also
46 lyse *M. tuberculosis* H37Rv. We present detailed analysis of one of these
47 mycobacteriophage (PDRPv). TEM and PCR analysis (of a conserved region within
48 the TMP gene) shows PDRPv to belong to *Siphoviridae* family and B1 sub-cluster,
49 respectively. The genome (69110 bp) of PDRPv is circularly permuted double-
50 stranded DNA with ~66% GC content and has 106 open reading frames (ORFs). On
51 the basis of sequence similarity and conserved domains, we have assigned function to
52 28 ORFs and have broadly categorized them into six groups that are related to
53 replication and genome maintenance, DNA packaging, virion release, structural
54 proteins, lysogeny related genes and endolysins.

55 The present study reports the occurrence of novel anti-mycobacterial phages in India
56 and highlights their potential to contribute to our understanding of these phages and
57 their gene products as potential antimicrobial agents.

58 **Keywords:** Mycobacteriophage, B1 sub-cluster, *M. tuberculosis*, phage diversity.

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61 INTRODUCTION

62 The occurrence of multi drug-resistant and extremely drug-resistant strains of *M.*
63 *tuberculosis* and a slow progress in finding new drugs for the treatment of
64 tuberculosis has created an alarming situation, which mandates the need for finding
65 alternate solutions to manage the disease. Mycobacteriophages are bacteriophages
66 that infect mycobacterial hosts including clinically important *M. tuberculosis* and the
67 non-pathogenic *M. smegmatis*. Phages have contributed significantly towards gaining
68 knowledge of their host (Mtb) and the tuberculosis disease. Their natural ability to
69 kill *Mycobacterium* spp has attracted many research groups to investigate their
70 possible role in the treatment/diagnosis of tuberculosis (Jacobs et al. 1993; Riska et
71 al. 1997; Chatterjee et al. 2000; Broxmeyer et al. 2002; Danelishvili et al. 2006).
72 Phages can be readily isolated from environmental samples such as soil, water and
73 sewage. Their highly prevalent and benign presence in the environment suggests
74 them to not have adverse effects on humans. Also, since bacteriophages are host-
75 specific and the fact that free exchange of genetic material is mostly not observed
76 amongst phages specific to different bacterial hosts further makes them safe for use in
77 humans. The first mycobacteriophage with *M. smegmatis* as host was isolated in 1947
78 (Gardner 1947) and a mycobacteriophage against *M. tuberculosis* was first
79 discovered in 1954 (Froman et al. 1954). During the 1960s and 1970s, phages were
80 used for typing of clinical strains of *M. tuberculosis* in various epidemiological
81 studies (McNerney 1999) in Europe and Asian countries such as India and Hong
82 Kong. Phage therapy has been popular in east European countries but with the

83 discovery of antibiotics and later with the advent of new molecular techniques,
84 interest in bacteriophages for the treatment of bacterial infections gradually faded
85 away. However with the emergence of drug resistant strains of pathogenic bacteria,
86 interest in phages is rising again as evident from the reports on discovery and
87 applications of mycobacteriophages (Jacobs et al. 1993; Riska et al. 1997; Chatterjee
88 et al. 2000; Broxmeyer et al. 2002; Danelishvili et al. 2006; Pope et al. 2011).
89 According to the records available on Actinobacteriophage database (PhagesDB.org),
90 about 9668 mycobacteriophages have been reported worldwide, amongst which, the
91 genomes of 1553 have been sequenced. The actinobacteriophage database
92 (Phagesdb.org) classifies mycobacteriophages based on nucleotide sequence
93 similarity determined by dot-plot analysis, average nucleotide identities, gene content
94 and pairwise genome analysis. To date, 27 clusters of mycobacteriophages have been
95 described on the database. The clusters are further subdivided into sub clusters. Some
96 phages do not group with any of the clusters and have been classified as singletons.
97 Mycobacteriophages have mosaic genomes and show a continuum of genetic
98 diversity across clusters and sub-clusters (Hatfull 2014; Pope et al. 2015). The
99 database shows the B1 sub-cluster to contain the largest number of
100 mycobacteriophages, followed by the A1 sub-cluster.

101 India has amongst the highest occurrence of tuberculosis infection rates in the world
102 and the widespread presence of the mycobacterial host suggests a high prevalence of
103 mycobacteriophages in the environment. However, except for a few studies (Pope et

al. 2011; Pope et al. 2015), not many reports have been documented on mycobacteriophages discovered from within the country. We believe that finding novel mycobacteriophages would contribute in expanding the existing database, and can lead to identification of un-explored virulent phages as a source of hydrolytic enzymes such as Endolysins, EPS depolymerase and Phospholipases/Esterases. Endolysins (Lysin A and Lysin B) that aid in the release of phage particles from the bacterial host by hydrolysis of the peptidoglycan component of the bacterial cell wall (Fischetti 2008; Schuch et al. 2013; Endersen et al. 2015) are being researched for their applications in diagnosis and as anti-bacterial agents.

In this study, we report the discovery of 17 mycobacteriophages isolated from 140 environmental samples, collected from various parts of the country (mostly from Delhi-NCR). Importantly, nine of these phages also infect and kill *M. tuberculosis* H37Rv. All nine phages were found to belong to *Siphoviridae* family and seven phages belonged to B1 sub-cluster.

MATERIALS AND METHODS

Isolation and purification of mycobacteriophages

We have given our phage discovery program an acronym called PDRP (phage discovery research project). A total of 140 samples (soil and water) were taken from a total of 50-55 locations which include Delhi and its adjoining areas and the travel destinations of the authors during the period of this study, which happened to be largely in the northern part of the country (Fig. 1). The samples were screened for the

presence of mycobacteriophages, using *M. smegmatis* mc²155 as the host. Screening is done on *M. smegmatis*, which is a non-pathogenic, fast growing species of Mycobacterium with a doubling time of 3-4 hours. Isolation of phages was carried out as described in previous reports (Pope et al. 2011) with slight modifications. Mycobacteriophages were isolated from the soil samples using phage buffer (10 mM Tris-HCl pH 7.5, 10 mM MgSO₄, 68.5 mM NaCl and 1 mM CaCl₂). The soil extract was centrifuged at 10,000 rpm for 10 minutes followed by filtration of the supernatant through 0.22 µm filters. For infection of bacterial cells, 50 µl of soil filtrate was added to 0.5 ml of *M. smegmatis* cells (grown to mid-log phase) and infection was allowed for 30 minutes at room temperature. Infected *M. smegmatis* cells were mixed with 4.5 ml of Middlebrook 7H10 top Agar (MBTA) containing 0.1% CaCl₂ and were poured over a plate of Middlebrook 7H10 agar containing Carbenicillin (50 µg/ml) and Cycloheximide (10 µg/ml). Culture plates were incubated at 37°C for up to 48 hours for plaques to appear. Phages were picked from the plaques and the infection of *M. smegmatis* was repeated several times to get purified mycobacteriophages. Once a pure mycobacteriophage preparation was obtained as evident from its distinct plaque morphology, amplification was done so as to collect high titer of the purified phage. The mycobacteriophages that were discovered from local environmental samples were named as PDRPi to PDRPxv, Shazeb, SimranZ1 and their information has been submitted to the phage database (PhagesDB.org).

Analysis of phage morphology by TEM

For TEM analysis, the high titer phage preparation was submitted to Advanced Instrumentation and Research Facility (AIRF), JNU, New Delhi. Morphology of the phages was characterized using a Jeol JEM-2100F transmission electron microscope (TEM). The samples were stained with 1% uranyl acetate and images were taken at a magnification of 30,000X.

Lytic activity of mycobacteriophages against *M. tuberculosis*

The purified phages were tested for their lytic activity against *M. tuberculosis* H37Rv in the laboratory of Dr. Mandira Basil Verma at the Patel Chest Institute, University of Delhi by standard spot test (Rybniker 2006). Briefly, 0.5 ml of *M. tuberculosis* H37Rv cells (from fresh late log phase culture) were added to 4.5 ml of top agar containing Oleic acid/Albumin/Dextrose/Catalase (OADC) and 1 mM CaCl₂ and poured onto 7H10-OADC agar plates containing Carbenicillin (50 µg/ml), Cycloheximide (10 µg/ml) and 1 mM CaCl₂. For the spot test, 10 µl of the phage suspension (prepared in the phage buffer) with pfu/ml in the range of 10⁶ to 10⁸ were pipetted onto the designated section on the bacterial lawn in the Petri plate and the spots were allowed to dry completely. Plates were then incubated for 21 days at 37 °C and were observed for the zone of lysis, post incubation. All experiments were performed in triplicate.

PCR amplification of a conserved region of Tape Measure Protein (TMP) gene for sub-cluster classification

The genomic DNA from purified phages was isolated using Wizard DNA cleanup kit (Promega) and used as the template for PCR amplification of TMP gene with sub cluster-specific primers as described in Smith et al. (2013). The primers (B1_CR Forward: 5'-AAA GGT GAT CGT GCC CAT CG-3' and B1_CR Reverse: 5'-GAA CCT CGT GAA CAG GTC GG-3') for B1 sub-cluster analysis were synthesized at Integrated DNA Technologies, India. The PCR was set up using Taq DNA Polymerase (Merck Biosciences) and the composition of PCR reaction was: reaction buffer (1x), dNTPs (0.25 mM), forward and reverse primers (5 pmol each), template DNA (50 ng) and Taq DNA polymerase (0.5 units) in a final volume of 20 µl. PCR amplification was carried out using Eppendorf Gradient Master-cycler and the conditions were: initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds followed by final extension at 72 °C for 5 min. The PCR products were analysed by agarose gel (1.2%) electrophoresis and the size of the amplicon was approximated using 100 bp DNA ladder (SM0323, Thermofischer scientific, USA). The gel image was recorded with Alpha imager gel documentation system.

Whole genome sequencing and analysis

PDRPv genomic DNA was isolated using Wizard DNA cleanup kit (Promega) and was sequenced at Lifecode technologies Pvt. Ltd., New Delhi using Illumina Hiseq platform. The sequencing data was subjected to quality check using FastQC. To analyze the sequence homology of PDRPv with other mycobacteriophages, BLASTn

program was used (Altschul et al. 1990). Open reading frames (ORF) were identified using DNAmaster tool (<http://cobamide2.bio.pitt.edu>) and were annotated using Blastp, Interproscan5 and NCBI CDD search tools. Phylogenetic analysis was performed using MEGA 7 software.

RESULTS

Isolation and purification of 17 mycobacteriophages

For isolation of mycobacteriophages, 140 environmental samples were collected largely from Delhi-NCR and from other parts of the country (Fig 1). Seventeen samples tested positive for the presence of mycobacteriophages, using *M. smegmatis* mc²155 as the bacterial host. Out of the 17 phages, 9 (PDRPi, PDRPii, PDRPiii, PDRPv, PDRPvi, PDRPix, PDRPxv, Shazeb, SimranZ1) exhibited distinct plaque morphology and hence were studied further (Table 1 and Fig 2). The size of plaques for each phage is given in table 1.

Morphology of mycobacteriophage TEM analysis of the phages revealed binal symmetry with an icosahedral head and a helical, non-contractile tail (Fig 3) that are characteristic features of the *Siphoviridae* family. The head and tail dimensions of the virions are given in table 1.

Lytic activity against *M. tuberculosis*

Phages, PDRPi, PDRPii, PDRPiii, PDRPv, PDRPvi, PDRPix, PDRPxv, Shazeb, and SimranZ1 were tested for lytic activity against *M. tuberculosis* by spot test. All the

211 nine phages exhibited lytic activity against *M. tuberculosis* H37Rv (Fig 4). We chose
212 one of these phages (PDRPv) for further characterization.

213 **Genome sequence analysis of PDRPv**

214 The genome size of PDRPv was found to be 69110 bp with 66.35 % GC-content and
215 was predicted to have a total of 106 ORFs. Twenty-eight of the coded proteins have
216 been assigned functions based on significant similarity with annotated proteins of
217 mycobacteriophages previously reported in databases and on the basis of the presence
218 of conserved domains. These can be broadly categorized into six groups related to
219 replication and genome maintenance (Gp6, Gp55, Gp62, Gp65, Gp74, Gp75 and
220 Gp89), DNA packaging (Gp1, Gp2 and Gp8), virion release (Gp51 and Gp52),
221 structural proteins (Gp10, Gp12, Gp18, Gp25, Gp28, Gp29, Gp30, Gp31, Gp32 and
222 Gp33), lysogeny-related genes (Gp47 and Gp68) and others (Gp78, Gp80, Gp85 and
223 Gp97). A summary of hypothetical proteins based on sequence homology is given in
224 Table 2 and their relative positions are mapped in Fig 5. The annotated genome
225 sequence of PDRPv has been submitted in GenBank with accession number
226 KR029086. By genome sequence comparison, PDRPv has been classified as
227 belonging to the Order- *Caudovirales*; the Family-*Siphoviridae*; the subfamily-
228 *Bclavirinae*; and Genus-*Pg1virus*.

229 **Cluster classification of the anti-*M. tuberculosis* phages**

230 Cluster relationship in phages can be established by a single gene analysis as
231 suggested by an interesting study (Smith et al. 2013), where conserved regions

present within tape measure protein (TMP), the longest gene in mycobacteriophages, were used for classification of phages into clusters and sub-clusters. Since plaque and virion morphology of many of the phages in this study (table 1) were observed to be similar to those of mycobacteriophages reported to belong to B1 sub-cluster and also because PDRPv genome aligned significantly with phage Oline of B1 sub-cluster, we chose to use the B1 sub-cluster specific primers for classification of the other eight phages (PDRPi, PDRPii, PDRPiii, PDRPvi, PDRPix, PDRPxv, Shazeb, SimranZ1). On PCR amplification, seven phages (except for PDRPii and SimranZ1) were identified to belong to the B1 sub-cluster on the basis of the size (493 bp) of the amplicon (Fig 6).

Comparative genomic study showed PDRPv to have close sequence homology (>95%) with phage Oline (GenBank: NC_023711.1) and phage Osmaximus (GenBank: JN006064.1). BLASTn analysis also showed homology between the putative TMP gene (5979 bp) from PDRPv and the TMP gene from mycobacteriophage Oline (Accession no.: JN192463.1, Identity: 100%, E-value: 0.0) and mycobacteriophage Vortex (Accession no.: JF704103.1, Identity: 99%, E-value: 0.0). Oline, Osmaximus and Vortex mycobacteriophages have also been reported to belong to B1 sub-cluster (Hatfull 2012a) and Both Oline and Osmaximus belong to genus *PgIvirus*.

Phylogenetic Analysis of PDRPv

Phylogenetic tree was constructed using the TMP amino acid sequence to determine

the relatedness of PDRPv with other members of the B cluster of mycobacteriophages and with D29, one of the most studied mycobacteriophage. As seen in the whole genome sequence alignment, PDRPv is very closely related to phage Oline of B1 sub-cluster (Fig 7). Also, it is interesting to note that B1 sub-cluster is more closely related to B4 than the other B sub-clusters. Mycobacteriophage D29, belongs to A2 cluster and is very distant from PDRPv.

DISCUSSION

To utilize the potential of phages in managing the mycobacterial diseases, it's important to find them in large numbers and to study i) their diversity, ii) specificity of their interactions with the host and iii) purify lytic. Discovery of mycobacteriophages gained momentum in the last few decades largely due to efforts such as SEA-PHAGES (Jordan et al. 2014) by Hatfull and team where a large number of undergraduates and school students participated in phage projects. To date, more than 1,553 mycobacteriophages have been isolated from various parts of the world, though only one report mentions a mycobacteriophage from India (Kumar et al. 2008). Hence, to discover the local phages, we collected 140 soil and water samples from various locations in the country. A total of 17 phages were found using *M. smegmatis* mc²155 as the bacterial host. *M. smegmatis* was used as a surrogate host for the initial screening of samples for isolating mycobacteriophages against *M. tuberculosis*, because being a non-pathogenic bacterium it can be used safely, and it grows relatively fast and hence saves time. The discovered phages were named is

274 based on the name of the project (**Phage Discovery Research Project**, PDRP) except
275 for Shazeb and SimranZ1 which were discovered through another project. Nine
276 (PDRPi, PDRPii, PDRPiii, PDRPv, PDRPvi, PDRPix, PDRPxv, Shazeb, SimranZ1)
277 out of 17 phages showed distinct plaque morphology and hence were analysed
278 further. The fact that these 9 phages also showed lytic activity against *M.*
279 *tuberculosis*, is significant since data on the ability of most of the reported
280 mycobacteriophages to infect *M. tuberculosis* is scarce (Hatfull 2012b; Hatfull 2014).
281 The B1-subcluster is most populous of all mycobacteriophage sub clusters to date and
282 has a total of 182 mycobacteriophages as verified members. The phages in B1-
283 subcluster have an average genome size of 68.5 kb with 66.5% GC content and the
284 average number of ORFs is 100.1. All phages of B1-subcluster belong to the
285 *Siphoviridae* family of phages.

286 Discovery of new phages capable of infecting and lysing *M. tuberculosis* hold great
287 potential application as antimicrobial agents of future. However, phages also pose
288 certain inherent constraints. The pros and cons of phage therapy have been reviewed
289 by many groups and some of the limitations on their use in clinical practice are: i)
290 the possibility of immunological response triggered by phages when administered in
291 large doses in patients, leading to restriction on using a particular phage more than
292 once, ii) high clearance rate of phages from the body and iii) inaccessibility of the
293 intracellular pathogens to phages (Carlton 1999; Brüssow 2005; Henein 2013;
294 Nilsson 2014). Extensive research and clinical trials using mycobacteriophages for
295 phage therapy may yield answer to the raised concerns, however the fact that the

296 phage therapy is indeed practiced with high success rate in certain countries (The
297 Phage Therapy Center at Tbilisi in Georgia is one such example), does promise it to
298 be an effective alternative/complement to antibiotics (Kutter et al. 2010). Recently, in
299 a promising study, Schooley et al. (2017) have effectively shown successful
300 bacteriophage therapy in the treatment of a patient infected with multi drug resistant
301 *A. baumannii*.

302
303 Such reports are very encouraging. However, since phages are an inexpensive natural
304 resource, they might not be a lucrative proposition to firms interested in making
305 proprietary products. Hence, it is incumbent on researchers, interested in finding
306 affordable solutions to infectious diseases, to explore and develop phages as effective
307 alternatives (McNerney and Traore 2005; Fu et al. 2015).

308 This preliminary study indicates high occurrence of mycobacteriophages in our
309 environment (about one in eight samples), highlighting the importance of discovering
310 and tapping the unexplored phages for tuberculosis and other infectious diseases as
311 well. To the best of our knowledge, this is the first report in recent years, where an
312 attempt is made to isolate and characterize mycobacteriophages from India. B1 sub-
313 cluster is the most populous sub-cluster, but most of the isolated phages are reported
314 from the USA. While this study has added to the existing repertoire of
315 mycobacteriophages, more studies are needed to discover and characterize the phage
316 diversity in India and study their anti-mycobacterial potential.

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327 CONFLICT OF INTEREST

328 The authors declare that they have no conflict of interest.

329 REFERENCES

- 330 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local
331 alignment search tool. *J. Mol. Biol.* **215**(3): 403–10. doi:10.1016/S0022-
332 2836(05)80360-2.
- 333 Broxmeyer, L., Sosnowska, D., Miltner, E., Chacón, O., Wagner, D., McGarvey, J., Barletta,
334 R.G., and Bermudez, L.E. 2002. Killing of *Mycobacterium avium* and *Mycobacterium*
335 *tuberculosis* by a mycobacteriophage delivered by a nonvirulent mycobacterium: a
336 model for phage therapy of intracellular bacterial pathogens. *J. Infect. Dis.* **186**(8):
337 1155–60. doi:10.1086/343812.
- 338 Brüssow, H. 2005. Phage therapy: The *Escherichia coli* experience. *Microbiology*,
339 **151**(7):2133-2140. doi:10.1099/mic.0.27849-0.
- 340 Carlton, R.M. 1999. Phage therapy: past history and future prospects. *Arch. Immunol. Ther.*
341 *Exp. (Warsz).* **47**(5): 267–274. doi:10.2217/fvl.15.3.
- 342 Chatterjee, S., Mitra, M., and Das Gupta, S.K. 2000. A high yielding mutant of
343 mycobacteriophage L1 and its application as a diagnostic tool. *FEMS Microbiol. Lett.*
344 **188**(1): 47–53. doi:10.1016/S0378-1097(00)00206-8.
- 345 Danelishvili, L., Young, L.S., and Bermudez, L.E. 2006. In vivo efficacy of phage therapy

- for *Mycobacterium avium* infection as delivered by a nonvirulent mycobacterium. Microb. Drug Resist. **12**(1): 1–6. doi:10.1089/mdr.2006.12.1.
- Endersen, L., Guinane, C.M., Johnston, C., Neve, H., Coffey, A., Paul Ross, R., McAuliffe, O., and O'Mahony, J. 2015. Genome analysis of Cronobacter phage vB_CsaP_Ss1 reveals an endolysin with potential for biocontrol of Gram-negative bacterial pathogens. J. Gen. Virol. **96**(2): 463–477. doi:10.1099/vir.0.068494-0.
- Fischetti, V.A. 2008. Bacteriophage lysins as effective antibacterials. Curr. Opin. Microbiol. **11**(5): 393–400. doi:10.1016/j.mib.2008.09.012.
- Froman, S., Will, D.W., and Bogen, E. 1954. Bacteriophage active against virulent *Mycobacterium tuberculosis*. I. Isolation and activity. Am. J. Public Health, **44**(10): 1326–1333. doi:10.2105/AJPH.44.10.1326.
- Fu, X., Ding, M., Zhang, N., and Li, J. 2015. Mycobacteriophages: An important tool for the diagnosis of *Mycobacterium tuberculosis* (Review). Mol. Med. Rep. **12**(1): 13–19. doi:10.3892/mmr.2015.3440.
- Gardner, G.M., and Weiser, R. S. 1947. A Bacteriophage for *Mycobacterium smegmatis*. Proc Soc Exp Biol Med **66**(1): 205. doi:doi: 10.3181/00379727-66-16037.
- Hatfull, G.F. 2012a. Complete Genome Sequences of 138 Mycobacteriophages. J. Virol. **86**(4): 2382–2384. doi:10.1128/JVI.06870-11.
- Hatfull, G.F. 2012b. The Secret Lives of Mycobacteriophages. In Advances in Virus Research, **82**: 179–288. doi:10.1016/B978-0-12-394621-8.00015-7.
- Hatfull, G.F. 2014. Mycobacteriophages: Windows into Tuberculosis. PLoS Pathog. **10**(3): 12–14. doi:10.1371/journal.ppat.1003953.
- Henein, A. 2013. What are the limitations on the wider therapeutic use of phage? Bacteriophage, **3**(June): e24872. doi:10.4161/bact.24872.
- Jacobs, W., Barletta, R., Udani, R., Chan, J., Kalkut, G., Sosne, G., Kieser, T., Sarkis, G., Hatfull, G., and Bloom, B. 1993. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. Science (80). **260**(5109): 819–822. doi:10.1126/science.8484123.
- Jordan, T.C., Burnett, S.H., Carson, S., Caruso, S.M., Clase, K., DeJong, R.J., Dennehy, J.J., Denver, D.R., Dunbar, D., Elgin, S.C.R., Findley, A.M., Gissendanner, C.R., Golebiewska, U.P., Guild, N., Hartzog, G.A., Grillo, W.H., Hollowell, G.P., Hughes, L.E., Johnson, A., King, R.A., Lewis, L.O., Li, W., Rosenzweig, F., Rubin, M.R., Saha, M.S., Sandoz, J., Shaffer, C.D., Taylor, B., Temple, L., Vazquez, E., Ware, V.C., Barker, L.P., Bradley, K.W., Jacobs-Sera, D., Pope, W.H., Russell, D.A., Cresawn, S.G., Lopatto, D., Bailey, C.P., and Hatfull, G.F. 2014. A broadly implementable research course in phage discovery and genomics for first-year undergraduate students. MBio, **5**(1): e01051-13. doi:10.1128/mBio.01051-13.
- Kumar, V., Loganathan, P., Sivaramakrishnan, G., Kriakov, J., Dusthakeer, A., Subramanyam, B., Chan, J., Jacobs, W.R., and Paranjy Rama, N. 2008. Characterization of temperate phage Che12 and construction of a new tool for diagnosis of tuberculosis. Tuberculosis, **88**(6): 616–623. doi:10.1016/j.tube.2008.02.007.
- Kutter, E., De Vos, D., Gvasalia, G., Alavidze, Z., Gogokhia, L., Kuhl, S., and Abedon, S.T. 2010. Phage therapy in clinical practice: treatment of human infections. Curr. Pharm. Biotechnol. **11**(1): 69–86. doi:10.2174/138920110790725401.
- McNerney, R. 1999. TB: The return of the phage. A review of fifty years of mycobacteriophage research. Int. J. Tuberc. Lung Dis. **3**(3): 179–184.

- McNerney, R., and Traore, H. 2005. Mycobacteriophage and their application to disease control. *J. Appl. Microbiol.* **99**(2): 223–233. doi:10.1111/j.1365-2672.2005.02596.x.
- Nilsson, A.S. 2014. Phage therapy—constraints and possibilities. *Ups. J. Med. Sci.* **119**(2): 192–198. doi:10.3109/03009734.2014.902878.
- Pope, W.H., Bowman, C. a, Russell, D. a, Jacobs-Sera, D., Asai, D.J., Cresawn, S.G., Jacobs, W.R., Hendrix, R.W., Lawrence, J.G., and Hatfull, G.F. 2015. Whole genome comparison of a large collection of mycobacteriophages reveals a continuum of phage genetic diversity. *Elife*, **4**: e06416. doi:10.7554/eLife.06416.
- Pope, W.H., Jacobs-Sera, D., Russel, D.A., Peebles, C.L., Al-Atrache, Z., Alcoser, T.A., Alexander, L.M., Alfano, M.B., Alford, S.T., Amy, N.E., Anderson, M.D., Anderson, A.G., Ang, A.A.S., Manuel, A., Barber, A.J., Barker, L.P., Barrett, J.M., Barshop, W.D., Bauerle, C.M., Bayles, I.M., Belfield, K.L., Best, A.A., Agustin, B., Bowman, C.A., Boyer, C.A., Bradley, K.W., Bradley, V.A., Broadway, L.N., Budwal, K., Busby, K.N., Campbell, I.W., Campbell, A.M., Carey, A., Caruso, S.M., Chew, R.D., Cockburn, C.L., Cohen, L.B., Corajod, J.M., Cresawn, S.G., Davis, K.R., Deng, L., Denver, D.R., Dixon, B.R., Ekram, S., Elgin, S.C.R., Engelsen, A.E., English, B.E. V, Erb, M.L., Estrada, C., Filliger, L.Z., Findley, A.M., Forbes, L., Forsyth, M.H., Fox, T.M., Fritz, M.J., Garcia, R., George, Z.D., Georges, A.E., Gissendanner, C.R., Goff, S., Goldstein, R., Gordon, K.C., Green, R.D., Guerra, S.L., Guiney-Olsen, K.R., Guiza, B.G., Haghighat, L., Hagopian, G. V., Harmon, C.J., Harmson, J.S., Hartzog, G.A., Harvey, S.E., He, S., He, K.J., Healy, K.E., Higinbotham, E.R., Hildebrandt, E.N., Ho, J.H., Hogan, G.M., Hohenstein, V.G., Holz, N.A., Huang, V.J., Hufford, E.L., Hynes, P.M., Jackson, A.S., Jansen, E.C., Jarvik, J., Jasinto, P.G., Jordan, T.C., Kasza, T., Katelyn, M.A., Kelsey, J.S., Kerrigan, L.A., Khaw, D., Kim, J., Knutter, J.Z., Ko, C.C., Larkin, G. V., Laroche, J.R., Latif, A., Leuba, K.D., Leuba, S.I., Lewis, L.O., Loesser-Casey, K.E., Long, C.A., Javier Lopez, A., Lowery, N., Lu, T.Q., Mac, V., Masters, I.R., Mccloud, J.J., McDonough, M.J., Medenbach, A.J., Menon, A., Miller, R., Morgan, B.K., Ng, P.C., Nguyen, E., Nguyen, K.T., Nguyen, E.T., Nicholson, K.M., Parnell, L.A., Peirce, C.E., Perz, A.M., Peterson, L.J., Pferdehirt, R.E., Philip, S. V., Pogliano, K., Pogliano, J., Polley, T., Puopolo, E.J., Rabinowitz, H.S., Resiss, M.J., Rhyan, C.N., Robinson, Y.M., Rodriguez, L.L., Rose, A.C., Rubin, J.D., Ruby, J.A., Saha, M.S., Sandoz, J.W., Savitskaya, J., Schipper, D.J., Schnitzler, C.E., Schott, A.R., Bradley Segal, J., Shaffer, C.D., Sheldon, K.E., Shepard, E.M., Shepardson, J.W., Shroff, M.K., Simmons, J.M., Simms, E.F., Simpson, B.M., Sinclair, K.M., Sjöholm, R.L., Slette, I.J., Spaulding, B.C., Straub, C.L., Stukey, J., Sughrue, T., Tang, T.Y., Tatyana, L.M., Taylor, S.B., Taylor, B.J., Temple, L.M., Thompson, J. V., Tokarz, M.P., Trapani, S.E., Troum, A.P., Tsay, J., Tubbs, A.T., Walton, J.M., Wang, D.H., Wang, H., Warner, J.R., Weisser, E.G., Wendler, S.C., Weston-Hafer, K.A., Whelan, H.M., Williamson, K.E., Willis, A.N., Wirtshafter, H.S., Wong, T.W., Wu, P., Yang, Y.J., Yee, B.C., Zaidins, D.A., Zhang, B., Z??niga, M.Y., Hendrix, R.W., and Hatfull, G.F. 2011. Expanding the diversity of mycobacteriophages: Insights into genome architecture and evolution. *PLoS One*, **6**(1): e16329. doi:10.1371/journal.pone.0016329.
- Riska, P.F., Jacobs, W.R., Bloom, B.R., McKittrick, J., and Chan, J. 1997. Specific identification of *Mycobacterium tuberculosis* with the luciferase reporter mycobacteriophage: use of p-nitro-alpha-acetylamino-beta-hydroxy propiophenone. *J. Clin. Microbiol.* **35**(12): 3225–3231.

- Rybniker, J. 2006. Host range of 14 mycobacteriophages in *Mycobacterium ulcerans* and seven other mycobacteria including *Mycobacterium tuberculosis* - application for identification and susceptibility testing. J. Med. Microbiol. **55**(1): 37–42. doi:10.1099/jmm.0.46238-0.
- Schuch, R., Pelzek, A.J., Raz, A., Euler, C.W., Ryan, P.A., Winer, B.Y., Farnsworth, A., Bhaskaran, S.S., Stebbins, C.E., Xu, Y., Clifford, A., Bearss, D.J., Vankayalapati, H., Goldberg, A.R., and Fischetti, V.A. 2013. Use of a Bacteriophage Lysin to Identify a Novel Target for Antimicrobial Development. PLoS One, **8**(4): e60754. doi:10.1371/journal.pone.0060754.
- Smith, K.C., Castro-Nallar, E., Fisher, J.N., Breakwell, D.P., Grose, J.H., and Burnett, S.H. 2013. Phage cluster relationships identified through single gene analysis. BMC Genomics, **14**(1): 410. doi:10.1186/1471-2164-14-410.

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Figure Legends

Fig. 1 Map of India showing geographical locations (red dots) from where soil/water samples (used in the discovery of mycobacteriophages) were collected.

Fig. 2 Plaque morphology of mycobacteriophages. Mycobacteriophages were isolated from the collected soil/water samples using *M. smegmatis* cells as the bacterial host. Culture plates were incubated at 37°C for up to 48 hours for plaques to appear.

Fig 3. TEM analysis of mycobacteriophages. The scale bar of transmission electron micrograph (TEM) with uranyl acetate stain corresponds to 100 nm.

Fig 4. Lytic activity of nine mycobacteriophages against *Mycobacterium tuberculosis* H37Rv by spot test method. Ten-microliter of each phage preparation (pfu/ml in the range of 10^6 to 10^8) was spotted onto the mycobacterial lawn, and the spots were allowed to dry completely. Plates were incubated at 37 °C for 21 days and observed for zone of lysis.

Fig. 5 Genome (DNA) organization of PDRPv, drawn by CGView Server (Grant and Stothard 2008), showing genes transcribed from leading and lagging strand in a clockwise and anti-clockwise direction, respectively.

Fig. 6 PCR analysis of B1-subcluster specific conserved region (493 bp) in TMP gene. M- 100 bp DNA ladder, 1- no template control, 2- PDRPi, 3- PDRPiii, 4- PDRPv, 5- PDRPvi, 6- PDRPix, 7- PDRPxv, 8-Shazeb. Amplicon size 493 bp.

Fig. 7 Phylogenetic tree constructed from protein sequence of tape measure protein (TMP). PDRPv appears to be most closely related to phage Oline. The phylogenetic analysis confirms the association of PDRPv to B1 sub-cluster.

Fig. 1 Map of India showing geographical locations (red dots) from where soil/water samples (used in the discovery of mycobacteriophages) were collected.

135x123mm (300 x 300 DPI)

Fig. 2 Plaque morphology of mycobacteriophages. Mycobacteriophages were isolated from the collected soil/water samples using *M. smegmatis* cells as the bacterial host. Culture plates were incubated at 37°C for up to 48 hours for plaques to appear.

86x43mm (300 x 300 DPI)

Fig 3. TEM analysis of mycobacteriophages. The scale bar of transmission electron micrograph (TEM) with uranyl acetate stain corresponds to 100 nm.

84x71mm (300 x 300 DPI)

Fig 4. Lytic activity of nine mycobacteriophages against *Mycobacterium tuberculosis* H37Rv by spot test method. Ten-microliter of each phage preparation (pfu/ml in the range of 10⁶ to 10⁸) was spotted onto the mycobacterial lawn, and the spots were allowed to dry completely. Plates were incubated at 37 °C for 21 days and observed for zone of lysis.

70x42mm (300 x 300 DPI)

Fig. 5 Genome (DNA) organization of PDRPv, drawn by CGView Server (Grant and Stothard 2008), showing genes transcribed from leading and lagging strand in a clockwise and anti-clockwise direction, respectively.

143x98mm (300 x 300 DPI)

Fig. 6 PCR analysis of B1-subcluster specific conserved region (493 bp) in TMP gene. M- 100 bp DNA ladder, 1- no template control, 2- PDRPi, 3- PDRPiii, 4- PDRPv, 5- PDRPvi, 6- PDRPix, 7- PDRPxv, 8-Shazeb. Amplicon size 493 bp.

91x44mm (300 x 300 DPI)

Fig. 7 Phylogenetic tree constructed from protein sequence of tape measure protein (TMP). PDRPv appears to be most closely related to phage Oline. The phylogenetic analysis confirms the association of PDRPv to B1 sub-cluster.

71x77mm (300 x 300 DPI)

Table 1: Dimensions of the plaques and of the virions: PDRPi, PDRPii, PDRPiii, PDRPv, PDRPvi, PDRPix, PDRPxv, SimranZ1 and Shazeb. The plaque size (after 48 hours of incubation) was measured using a ruler and recorded. Ten plaques for each phage were measured and the average size is recorded. Data on the size of head & tail of each phage was obtained by TEM analysis.

S.No.	Phage Name	Plaque Size (Average $\pm$ Stdv) (mm)	Phage Size (Average $\pm$ Stdv)	
			Head (nm)	Tail (nm)
1	PDRPi	4.7 $\pm$ 0.82	54.5 $\pm$ 2.2	267 $\pm$ 1.6
2	PDRPii	2.1 $\pm$ 0.21	58.8 $\pm$ 0.9	284 $\pm$ 2.2
3	PDRPiii	4 $\pm$ 0.81	51.6 $\pm$ 1.0	291 $\pm$ 2.3
4	PDRPv	5.05 $\pm$ 0.28	57.6 $\pm$ 0.8	291 $\pm$ 2.5
5	PDRPvi	3.8 $\pm$ 0.54	56.9	297
6	PDRPix	2.45 $\pm$ 0.43	57.1 $\pm$ 0.2	293 $\pm$ 1.1
7	PDRPxv	4.65 $\pm$ 0.41	57.8	292
8	Shazeb	2.65 $\pm$ 0.47	45.8 $\pm$ 1.5	199 $\pm$ 5.9
9	SimranZ1	5 $\pm$ 0.28	47.1 $\pm$ 0.8	202 $\pm$ 0.5

Table 2. List of Open Reading Frames in PDRPv genome, with predicted functions.

S.no.	Position		(+/-)	Annotation	Protein signature database	
	Start	Stop			Conserved domain	Accession No
Gp1	1	567	+	Terminase small	Nucleoside triphosphate hydrolase	IPR027417
Gp2	564	2351	+	Terminase large	Terminase-like family	PF03237
Gp6	3361	3765	-	RuvC Resolvase	Crossover junction endodeoxyribonuclease RuvC	PF02075
Gp8	4438	6357	+	Portal protein		
Gp10	8927	9190	+	Minor head	Phage Mu protein F like protein	PF04233
Gp11	9304	11049	+	Scaffolding		
Gp12	11149	11946	+	Major capsid		
Gp18	15106	15336	-	Major tail subunit		
Gp25	18254	18676	+	Tail assembly chaperone		
Gp28	19816	25794	+	Tapemeasure protein		
Gp29	25804	27237	+	MTP		
Gp30	27234	28346	+	MTP		
Gp31	28343	30598	+	MTP		
Gp32	30602	31948	+	MTP		
Gp33	31952	33112	+	MTP		
Gp47	38772	39197	-	Repressor	HTH_XRE	cd00093
Gp51	40121	41410	+	Lysin A	Peptidase family M23; Amidase_2; Putative peptidoglycan binding domain	PF01551; PF01510; PF01471
Gp52	41420	42775	+	Lysin B	Alpha/Beta hydrolase fold	IPR029058
Gp55	44961	46757	-	Helicase	Helicase conserved C-terminal domain	PF00271
Gp62	48727	51474	-	Primase	AE Prim S like Superfamily	cl01287
Gp65	52426	54285	-	DNA polymerase	DNA pol A Superfamily	cl02626
Gp68	54840	54992	-	Restriction Directionality Factor (RDF)		

Gp74	57516	57740	+	HNH endonuclease		
Gp75	57737	58444	+	RNase	Homeodomain-like domain	PF13384
Gp78	59478	59606	+	Phospholipase A2	PLA2 like Superfamily	cl05417
Gp80	59987	60589	+	beta lactamase	Beta-lactamase-like	IPR012338
Gp85	61902	62165	-	TIM barrel	TIM phosphate binding Superfamily	cl21457
Gp89	63096	63398	-	HNH endonuclease	HNHc Superfamily	cl00083
Gp97	64887	65189	-	Transpeptidase	Sortase Superfamily	cl09098

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