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Isolation and Characterization of vB_MsmS_Celfi: A New *Mycobacterium tuberculosis* Bacteriophage

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Abstract

Introduction: Because of the clinical relevance of *Mycobacteria*, and from a therapeutic perspective, there is an increasing interest to study phages that infect bacteria belonging to this genus.
Materials and Methods: A phage was isolated from a soil sample, using *Mycobacterium smegmatis* as host. Its characterization included sequencing, annotation, and analysis of the genome, host range determination, and electron microscopy imaging.
Results: *Mycobacterium* phage vB_MsmS_Celfi is a temperate phage able to infect *Mycobacterium tuberculosis* with high efficiency. From electron microscopy images, Celfi belongs to the *Siphoviridae* family. Genome analysis classified phage Celfi into cluster L, subcluster L2 of Actinobacteriophage clusters. *Mycobacterium* phage Celfi exhibits a Lysin B distant to those present in other members of the subcluster and other mycobacteriophages.
Conclusions: The discovery of new phages that infect *M. tuberculosis* could contribute to the development of novel tools for detection systems and future treatment of the disease.

Keywords: *Mycobacterium* phage, LysB, *M. tuberculosis*

Introduction

Mycobacterial species are responsible for a wide variety of infectious diseases, including leprosy, caused by *Mycobacterium leprae*, and tuberculosis caused by *Mycobacterium tuberculosis*. Nontuberculous mycobacteria (NTM) lung diseases (including those caused by *Mycobacterium avium*, *Mycobacterium abscessus*, and *Mycobacterium fortuitum*) are also increasingly recognized as opportunistic infections in humans.[1,2] However, many mycobacteria are difficult to manipulate in laboratory conditions. For instance, *M. tuberculosis* requires incubation of 3 to 4 weeks at 37°C to form colonies, virulent strains have to be manipulated in a biosafety level III[3] and *M. leprae* can only propagate in footpads of mice or in nine-banded armadillos.[4] Owing to the relevance of members of this genus, mycobacteriophages, viruses that specifically infect *Mycobacterium* spp., are of special interest. Because many of them can replicate on *Mycobacterium smegmatis*, a fast-growing and easy-to-manipulate bacteria, mycobacteriophages and their proteins have become an attractive option to engineer mycobacterial genomes and for tuberculosis diagnosis.[5–11]

Most of the mycobacteriophages identified presently were isolated and sequenced in the context of the SEA-PHAGES (Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science) program. This program promotes the engagement of undergraduate students in research by the isolation and characterization of bacteriophages.[12–14]

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The success of this program was a source of inspiration to carry out an International Phage Course at the University of Buenos Aires directed toward graduate students and young researchers from Latin America. During this 2-week course, students were able to isolate, sequence, and annotate the genomes of different bacteriophages from environmental samples (commentary in this issue).

In this study, we present one of those phages that was isolated from a soil sample from the outside field of the School of Exact and Natural Sciences, University of Buenos Aires where the course took place.

Materials and Methods

Mycobacterium phage vB_MsmS_Celfi (phage Celfi) was isolated from a soil sample from Buenos Aires, Argentina. In brief, ~1 g of soil was incubated with phage buffer (10 mM Tris-HCl pH = 7.5; 68.5 mM NaCl; 10 mM $MgSO_4$; 1 mM $CaCl_2$), after vigorous mixing, the sample was centrifuged for 10 min at 4000 *g* and the supernatant was filtered using a 0.22 μm membrane. After enrichment in Middlebrock 7H9 medium supplemented with albumin dextrose complex (ADC) 10% (0.2% w/v dextrose; 0.085% w/v NaCl; 0.5% w/v bovine serum albumin) at 37°C with agitation at 250 rpm overnight at 37°C using *M. smegmatis* mc^2155 as host, isolation and purification were carried out through the double layer assay as described by Kropinski et al.[15] followed by five subsequent rounds of purification. High-titer stocks were prepared on solid media: 80 Petri dishes were incubated with a layer of top agar containing 100 μL of a culture of *M. smegmatis* mc^2155 in exponential phase of growth and 100 μL of phage suspension containing ~500–1000 plaque forming unit (PFU), sufficient to get confluent plaques but not complete lysis. Once plaques were obtained, plates were covered with phage buffer and incubated overnight at 4°C, then the top agar layer was scrapped, transferred to conical tubes, and centrifuged. The supernatant was filtered through a 0.22 μm membrane and concentrated by ultracentrifugation at 100,000 *g* for 3 h at 4°C.

Bacterial strains and culture conditions

M. smegmatis mc^2155 was used as the isolation host and was cultured in Middlebrock 7H9 media supplemented with ADC 10% at 37°C with agitation at 250 rpm. For solid culture, Middlebrock 7H10 was used.

M. tuberculosis mc^26230,[16] *Mycobacterium intracellulare*, *M. abscessus*, *M. avium*, and *Mycobacterium kansasii* were manipulated under biosafety level 2 (BSL-2) conditions. The NTM strains are clinical isolates. All of these strains were cultured in 7H9 media supplemented with oleic acid albumin dextrose complex Enrichment (Becton, Dickinson and Company, Sparks, MD) and PANTA (Becton, Dickinson and Company) at 37°C without agitation. In particular, for *M. tuberculosis* mc^26230, pantothenic acid (100 μg/mL) was added.

Because of the complex cell envelope, mycobacteria tend to form aggregates in liquid culture that are prevented by the addition of Tween 80 (0.05% v/v). Since this surfactant can abrogate mycobacteriophage infection, bacteria were cultured in the presence of Tween and extensively washed before infection.

Host range assay

To determine the host range, several mycobacteria species of clinical relevance were employed. Tenfold serial dilutions of the phage stock were performed in phage buffer. To do a spot test, 7H10 plates were poured with 5 mL of 7H10 top agar containing 100 μL of bacterial culture in exponential phage. Once the overlay solidified, 10 μL of each dilution was dispensed on the overlay. Plates were incubated at 37°C for 48 h in the case of *M. smegmatis* mc^2155 or for 2–8 weeks for the rest of the strains to allow the correct growth of the lawn and plaque visualization.

When plaques were evident, phage titer was calculated according to

$$\text{PFU/mL} = \text{number of plaques} / (\text{volume of dilution} \times \text{dilution factor}).$$

Efficiency of plating (EoP) was calculated as the titer of the phage in a stated bacterial strain/the maximum phage titer observed.

DNA isolation and sequencing

DNA was extracted from a high-titer phage stock using a simple phenol–chloroform protocol as described on the Phage Hunters program website.[17] DNA samples were sent for sequencing to a local facility. Genomic libraries were constructed using Nextera® XT DNA Sample Preparation Kit (Illumina) following the manufacturer's instructions. Individual libraries were indexed with Nextera XT Index Kit. Paired-end reads were obtained using Illumina MiSeq platform at the National Institute of Infectious Diseases ''Dr. Carlos G. Malbran'' (Buenos Aires, Argentina), which generated 133,300 250-bp paired-end raw reads. The adapter and low-quality sequences were trimmed, and short reads were filtered out using Trimmomatic software[18] v 0.36 using default configurations. The trimmed reads were *de novo* assembled using Velvet 1.2.10.[19]

Bioinformatic analysis and annotation

Sequence analysis and annotation were performed using DNAMaster[20] and curated manually. Start sites were found using Glimmer[21] and GeneMark[22]; tRNAs were predicted using Aragorn[23] and tRNA scan.[24,25] Putative functions were assigned using BLAST[26] and HHPred.[27] Alignments and evolutionary analyses were conducted in MEGA X.[28]The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model.[29]

Transmission electron microscopy

Phage morphology was assessed by transmission electron microscopy (TEM) in collaboration with the Laboratory of Food Microbiology at ETH Zurich. High titer phage stocks were negatively stained for 20 s with 2% uranyl acetate, pH 8.0, on carbon-coated copper grids (Quantifoil), and observed at 100 kV on a Hitachi HT 7700 scope equipped with an AMT XR81B Peltier cooled CCD camera (8M pixel). Only six suitable particles were measured for phage Celfi, and, therefore, only a range of size is provided.

Results

Mycobacterium phage vB_MsmS_Celfi (phage Celfi) was isolated after enrichment from a soil sample taken outside of the School of Exact and Natural Sciences, University of

Table 1. Host Range of *Mycobacterium* Phage Centro Latinoamericano de Formación Interdisciplinaria

Mycobacterium *spp.*	*EoP*
M. smegmatis mc^{2}155	$1.3\ 10^{-1}$
M. tuberculosis mc^{2}6230	1
M. intracellulare[a]	*
M. abscessus[a]	*
M. avium[a]	*
M. kansasii[a]	*

*Absence of individual plaques.
[a]Clinical isolates.
EoP, efficiency of plating.

Buenos Aires, Argentina (34° 32′ S, 58° 26′ W), using *M. smegmatis* mc^{2}155 as host.

Host range and morphology

Phage Celfi form plaques on *M. smegmatis* and *M. tuberculosis* strains, but not on the *M. intracellulare*, *M. abscessus*, *M. avium*, and *M. kansasii* strains tested (Table 1). Phage plaques on *M. smegmatis* mc^{2}155 were mostly clear, big, and round, with a diameter of 1.63 ± 0.28 mm (Fig. 1A); however, its EoP on *M. smegmatis* mc^{2}155 was $1.3\ 10^{-1}$ compared with the *M. tuberculosis* mc^{2}6230 strain.

Morphologically, phage Celfi is a Siphovirus. Virion particles of phage Celfi have an icosahedral head (head diameter range 61–69 nm) and a long noncontractile tail (tail length range 189–246 nm; Fig. 1B, C).

Sequencing and genome organization

Phage Celfi genome consists of a double-stranded DNA molecule of 77086 bp (125-fold coverage) with an overall guanine cytosine (GC) content of 59%. Genome analysis revealed 133 potential coding DNA sequences, 122 open-reading genes (ORFs), and 11 tRNA genes: 97 ORFs are transcribed rightward and 25 are transcribed leftward. The most commonly used start codon is ATG (50.4%), with lower usage of GTG (30.1%) and TTG (19.5%). The genome organization can be divided into the following modules: DNA packaging, virion structure, lysis, integration, immunity, and replication; a schematic representation is shown in Figure 2, and a list of predicted genes and gene products is available in Supplementary Table S1.

The sequences were deposited in GenBank under the accession number MT758688.

Comparative genome analysis

DNA sequence analysis indicated that phage Celfi belongs to the *Siphoviridae* family, *Bronvirus* genus, and to cluster L, subcluster L2 of Actinobacteriophage clusters.[13,14,30] Genomes of phages belonging to subcluster L2 have an average length of 75507 bp and GC% of 58.9. Average nucleotide sequence identity of phage Celfi with the other 25 phages in subcluster L2 is 92–94% (Ref.[31]). Interestingly, the closest match was with a prophage present in *Mycobacterium* sp. DL90 chromosome (NZ_CP034072.1) (93.9% identity). Even though *Mycobacterium* sp. DL90 genome has not been annotated, we were able to identify that the prophage is integrated into a putative tRNA gene. Phage Celfi, similar to other phages in the subcluster L2, encodes a tyrosine integrase. A putative *attP* site upstream the integrase gene was identified in the DNA of phage Celfi (pos 31626–31655), and a predictive *attB* site for phage Celfi in a $tRNA^{Lys}$ gene (coordinates 5834590–5834620 in *M. smegmatis* mc^{2}155 genome) was found. In addition to the integrase gene (*gene 37*), two transcriptional regulators (a putative immunity repressor, *gene 39*, and putative Cro protein, *gene 40*) and a putative excisionase (*gene 41*) could be identified in Celfi's genome. All these data suggest that phage Celfi is a temperate phage. In agreement with this, we spotted a few turbid plaques in lawns of *M. smegmatis* mc^{2}155. Sequence analysis comparison with two closely related phages (MkaliMitinis3 and Guuelad) and the *Mycobacterium* sp. DL90 prophage suggests that bacteriophage Celfi has cohesive ends with a common canonical cohesive site sequence: TCGATCAGCC; however, direct sequencing evidence falls four nucleotides short to cover the cohesive end site, which is expected due to the mechanisms of library construction. Further experiments and sequencing of the genome ends should be performed to corroborate this and report this sequence unequivocally.

As found in MkaliMitinis3 and Guuelad and also conserved in other *Syphoviridae* phages, it was possible to identify a sequence (GGGAAAGG) likely to generate a ribosomal slippage in ORF 14; after a -1 frameshift, two different products would

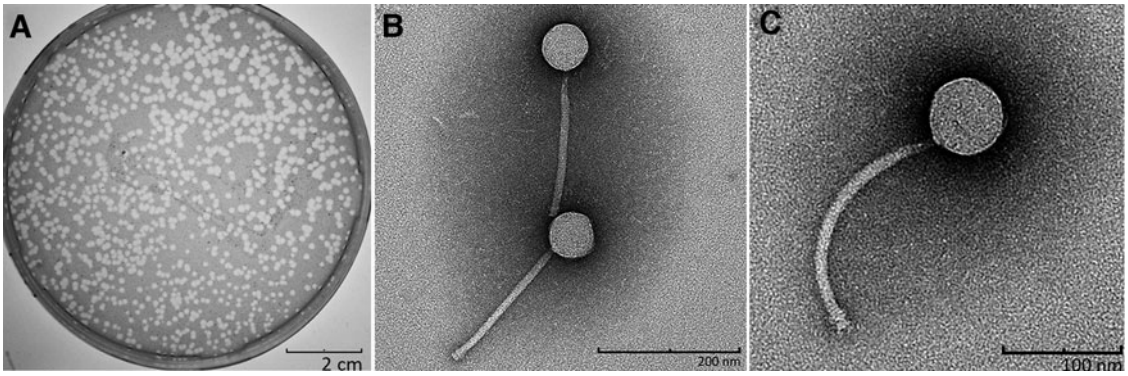

FIG. 1. *Mycobacterium* phage Celfi belongs to the *Siphoviridae* family. **(A)** Plaques of *Mycobacterium* phage CELFI on *Mycobacterium smegmatis* mc^{2}155. **(B)** and **(C)** TEM images of CELFI phage. Magnification 60.0 k. CELFI, Centro Latinoamericano de Formación Interdisciplinaria; TEM, transmission electron microscopy.

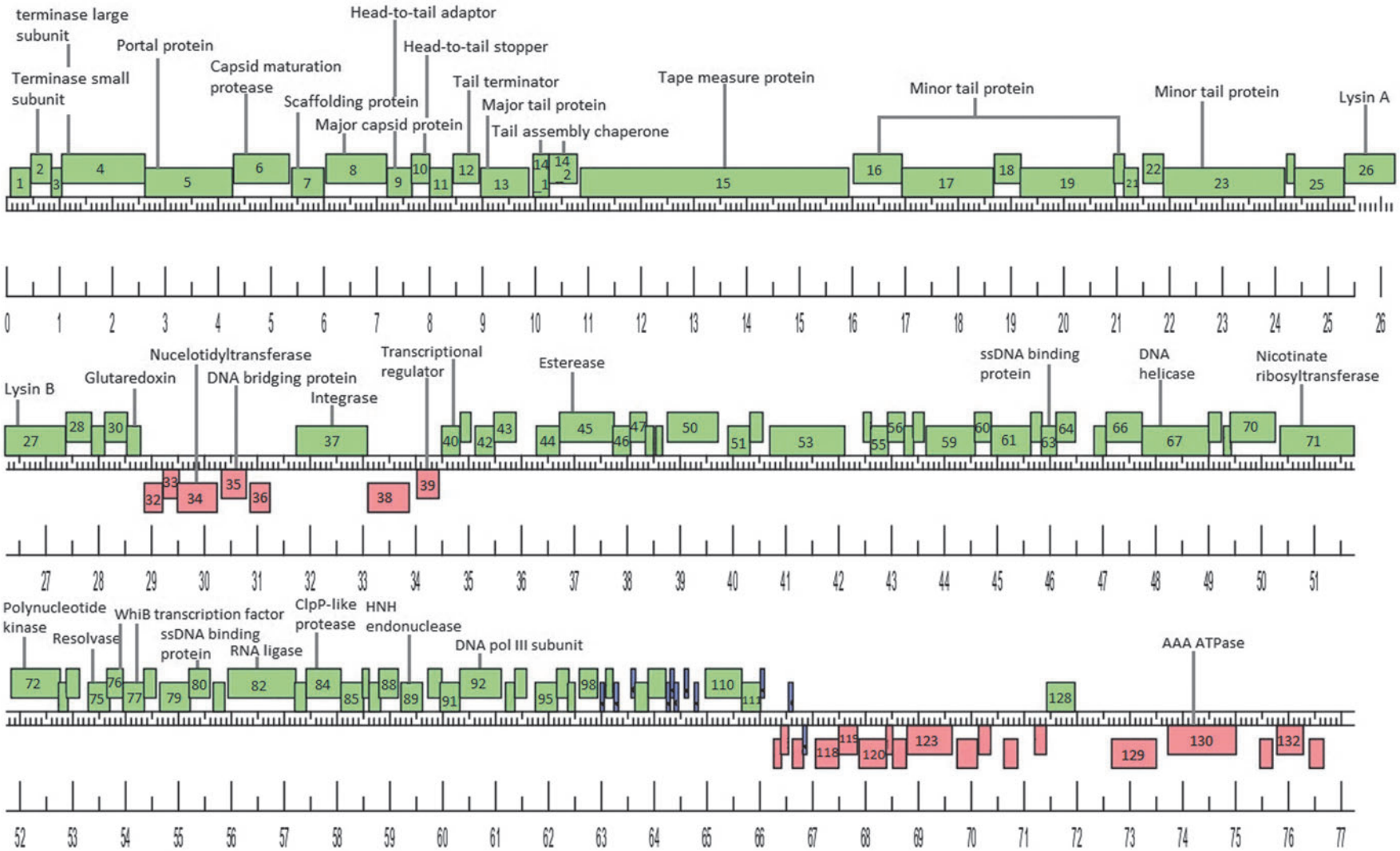

FIG. 2. Schematic representation of *Mycobacterium* phage CELFI genome. The viral genome is represented in three tiers, with markers spaced at 100 bp intervals. The predicted genes are shown as boxes either above or below the genome, depending on whether they are rightward (green boxes) or leftward (red boxes) transcribed. tRNAs are shown in blue. Gene numbers are shown within each box.

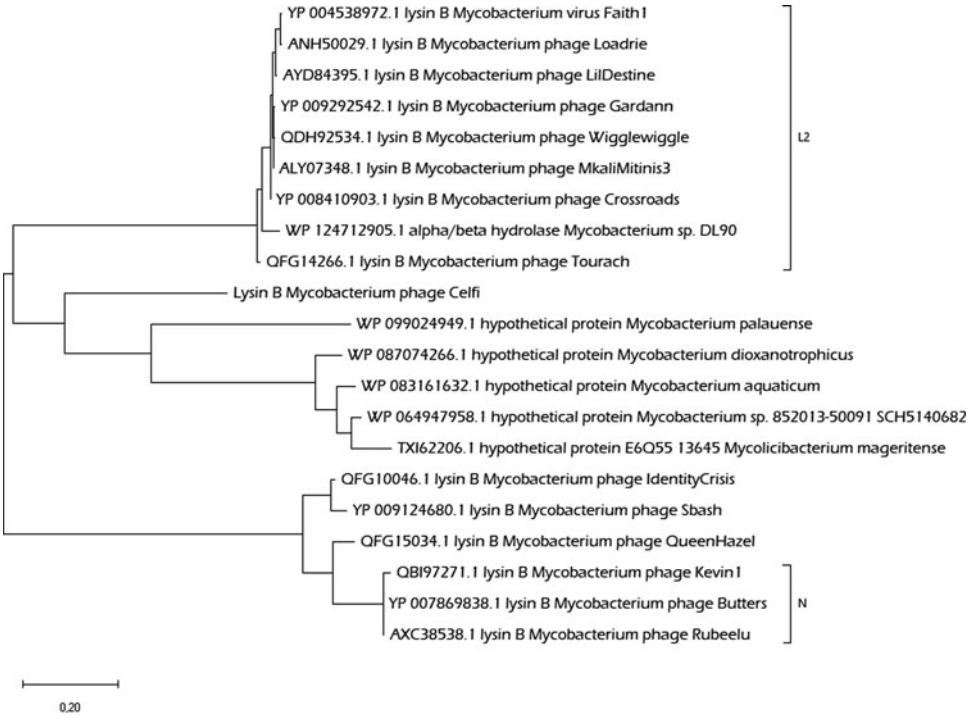

FIG. 3. Phylogenetic analysis of bacteriophage Celfi LysinB. The sequence of LysB was analyzed by BLASTp. The closest matches with an identity higher to 40% were aligned with MUSCLE and used to construct a phylogenetic tree in MEGA-X. The methods used were Maximum Likelihood and JTT matrix-based model.[24] The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

be synthesized, both predicted as tail assembly chaperones and depicted as 14_1 and 14_2 in Figure 2.

A notable difference between phage Celfi and the rest of the subcluster L2 phages, including the prophage integrated in the *Mycobacterium* sp. DL90 genome, was found in *gene 27* that codes for a putative lysin B (LysB). LysB enzymes are part of the lysis cassette in Mycobacteriophages, which cleave the mycolylarabinogalactan bond to release free mycolic acids and disrupt the outer membrane, facilitating the liberation of virions after infection.[32–34] *Gene 27* is 1152 bp long and the gene product is a 384 aa protein. At the nucleotide level, the first 223 bp is highly similar between phage Celfi and all subcluster L2 phages (85–89% identity), but the rest of the *lysB* gene did not match any nucleotide sequence in the database (data not shown). A protein Blast search, using gp27 sequence as a query, retrieved a series of mycobacterial hypothetical proteins and Mycobacteriophage LysB proteins with an amino acid identity $\sim$45%. By comparing all these sequences, a tree with the highest log likelihood (–5199.58) was obtained, which is shown in Figure 3. The tree shows that Celfi LysB is more related to hypothetical proteins present in *Mycobacterium* spp. than to other members of subcluster L2. We were only able to identify the protein present in *Mycolicibacterium mageritense* as part of a prophage encoded in the bacterial genome.

For the hypothetical mycobacterial proteins, sequence similarity with Celfi LysB was observed after the first 89 amino acids of the protein. On the contrary, for the mycobacteriophage LysB proteins, the highest conservation was observed at the N-terminal of the protein (Supplementary Fig. S1). Previous analyses of members of LysB proteins have predicted a PG-Binding Domain (pfam01471) within this region[30] but we did not identified one in gp27. Despite that, a serine (position 169), the GXP motif located $\sim$40 residues downstream, the aspartic acid (position 243), and a histidine (position 349) were conserved in gp27 and all the other compared proteins, being good candidates for the catalytic triad Ser–Asp–His previously described in other LysB and lipolytic enzymes[35,36] (Supplementary Fig. S1). Finally, using HHPred, we were able to identify a predicted structure similar to bacteriophage D29 LysB with high probability (100%), between amino acids 81 and 376.

This is a good example of the genetic diversity of these highly abundant entities and emphasizes why there is a very low probability of finding an identical copy of a previously characterized phage.

Conclusion

As of today, 11281 mycobacteriophages have been isolated of which, 1911 have been sequenced.[13,36] Despite that, their abundance and diversity allowed us to isolate a new bacteriophage.

Through bioinformatic analysis, it was possible to determine that mycobacteriophage Celfi belongs to cluster L, subcluster L2. This cluster is composed of 59 members and divided into four subclusters, L2 being the most populated.[13] As well as all of the members of this cluster, Mycobacteriophage Celfi probably exhibits a temperate life cycle with a genome length and GC content similar to the average of cluster L. When compared with its closest match, a prophage integrated in *Mycobacterium* sp. DL90, it was possible to identify the putative *attP* and *attB* in *M. smegmatis* mc^{2}155 genome. We were also able to predict a ribosomal slippage in the putative tail assembly chaperone gene, allowing the translation of two proteins with different lengths, a characteristic that is shared with members of the cluster and other Siphophages.

Interestingly, only a small proportion of the mycobacteriophages isolated in *M. smegmatis* infect *M. tuberculosis*, and most of them are restricted to a small number of clusters.[37]

Remarkably, this new mycobacteriophage infects *M. tuberculosis* with an apparent higher EoP than the host used for isolation. One difference with the other bacteriophages in cluster L was a quite dissimilar sequence for LysB, which cleaves the mycolylarabinogalactan bonds, facilitating progeny release. As shown in Figure 3, phage Celfi LysB is close to putative lipolytic proteins present in several mycobacterial species (including *Mycobacterium palauense*, *Mycobacterium dioxanotrophicus*, *Mycobacterium aquaticum*, and *Mycolicibacterium mageritense*), suggesting the possibility of alternative hosts for this phage in nature. Considering the complexity of the mycobacterial cell wall, the high EoP of Celfi in *M. tuberculosis* might be at least partially explained by the action of this particular LysB protein. The cotreatment of LysA and LysB to render *M. tuberculosis* susceptible to endolysin treatment has been proposed,[34] so further characterization of phage Celfi LysB activity could be interesting from a biotechnological perspective.

Considering the clinical relevance of *M. tuberculosis*, the isolation of new bacteriophages capable of infecting it broadens the spectrum of tools for genetic manipulation, diagnosis, and, may be in the future, for treatment of this complex human pathogen. In 2019, it was described for the first time the use of engineered mycobacteriophages to treat a patient with cystic fibrosis chronically infected with *M. abscessus.*[38] All of the phages used in the therapeutic cocktail had been isolated in the context of an educational program without the perspectives of therapeutic use, highlighting the impact that these projects can have at many different levels.

Authors' Contributions

Mycobacterium phage vB_MsmS_Celfi (phage Celfi) was isolated during the phage course 2016. Owing to time constraints, phage preparations were further purified, subjected to a cesium chloride gradient before electron microscopy (EM) analysis, and DNA extraction for sequencing. F.P., L.R.S., V.G., M.D.P., E.U., M.E.D., J.P.C., and F.Z. contributed to concepts/design, data acquisition/analysis/interpretation, and article preparation. M.D., P.G., and J.K. contributed to EM data acquisition/analysis and revising the article. E.S. and D.F.D.P. contributed to phage genome assembly. A.R. contributed to bioinformatics analysis, article preparation, and revising the article. R.R.R. contributed to revising the article. M.P. contributed to concepts/design, data analysis/interpretation, preparation, revising, and approval of the article. This article has been submitted solely to this journal and is not published, in press, or submitted elsewhere.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Table S1

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