

A Second Endolysin Gene Is Fully Embedded In-Frame with the *lysA* Gene of Mycobacteriophage Ms6

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Abstract

Mycobacteriophages are dsDNA viruses that infect mycobacterial hosts. The mycobacteriophage Ms6 accomplishes lysis by producing two cell wall hydrolytic enzymes, Lysin A (LysA) that possesses a central peptidoglycan recognition protein (PGRP) super-family conserved domain with the amidase catalytic site, that cleaves the amide bond between the *N*-acetylmuramic acid and L-alanine residues in the oligopeptide crosslinking chains of the peptidoglycan and Lysin B (LysB) a mycolylarabinogalactan esterase that hydrolyzes the mycolic acids from the mycolyl-arabinogalactan-peptidoglycan complex. Examination of the endolysin (*lysA*) DNA sequence revealed the existence of an embedded gene (*lysA*₂₄₁) encoded in the same reading frame and preceded by a consensus ribosome-binding site. In the present work we show that, even though *lysA* is essential for Ms6 viability, phage mutants that express only the longer (Lysin₃₈₄) or the shorter (Lysin₂₄₁) endolysin are viable, but defective in the normal timing, progression and completion of host cell lysis. In addition, both endolysins have peptidoglycan hydrolase activity and demonstrated broad growth inhibition activity against various Gram-positive bacteria and mycobacteria.

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Introduction

At the end of the replication cycle, bacteriophages must exit the host cell and disperse their newly formed progeny to infect new cells. The main barrier to host lysis is the peptidoglycan, a strong and stable structure that allows the bacterial envelope to withstand internal osmotic pressure [1]. With the exception of filamentous phages that as a result of their unique morphology and morphogenesis can extrude through the envelope without fatal consequences for the host, all other phages must either degrade or otherwise compromise the peptidoglycan to cause lysis [2,3]. Most of the tailed double-stranded DNA (dsDNA) phages achieve the proper time for lysis by the consecutive use of two lysis proteins – holin and endolysin. Holins are small hydrophobic membrane proteins that during the late phase of phage development progressively accumulate in the cytoplasmic membrane of the host and while the proton-motive force is maintained assemble into oligomers and rafts of intrinsic stability [2,4]. At a precise time programmed into its primary structure and upon a specific trigger event (holin effector concentration and partial depolarization of the membrane), the holin suddenly causes disruption of the membrane with non-specific hole formation and collapse of the membrane potential which sets the time of lysis by allowing the destruction of the cell wall by the released or activated phage encoded muralytic enzymes, the endolysins [5,6]. The term endolysin is used to describe the dsDNA bacteriophage-encoded peptidoglycan hydrolases, which are synthesized in phage-infected cells at the end of the multiplication cycle. They are characterized by the ability to directly target bonds in the peptidoglycan layer of

the bacterial cell wall; the result of this activity is degradation of the rigid murein layer and release of newly assembled virions by way of lysis [7,8]. Endolysins distribute between five major functional types; i) *N*-acetylmuramidases (lysozymes); ii) endo- β -*N*-acetylglucosaminidases, which all hydrolyze the β -1, 4 glycosidic bonds in the murein; iii) transglycosylases, which attack the same bonds but form a cyclic 1, 6 anhydro-*N*-acetylmuramic acid product; iv) *N*-acetyl-muramoyl-L-alanine amidases, which hydrolyze the amide bond between the *N*-acetylmuramic acid and L-alanine residues in the oligopeptide crosslinking chains; v) endopeptidases, which attack the peptide bonds in the same chains. In phages infecting Gram-positive bacteria, the most common architecture includes two clearly separated functional domains: the N-terminal domain that generally harbours the enzymatic activity, whereas the cell wall binding domain located at the C-terminal region directs the enzymes to their substrates and may restrain the enzyme lytic action to a particular type of cell wall [3,7]. Nonetheless, at least four bifunctional lysins have also been reported, consisting of an N-terminal and central catalytic domain with different specificity and a C-terminal substrate-binding domain; examples are the endolysins encoded by *Streptococcus agalactiae* bacteriophage B30 (muramidase and endopeptidase) [9], *Staphylococcus aureus* phage ϕ 11 (endopeptidase and amidase) [10], *Streptococcus agalactiae* phage NCTC 11261 (endopeptidase and muramidase) [11], and *Staphylococcus warneri* M phage ϕ WMY (endopeptidase and amidase) [12]. Most known endolysins lack signal peptide (SP) sequences and depend entirely on the cognate holins for release to the peptidoglycan. However, endolysins containing N-terminal secretory signals have already

been described. The endolysin of *Oenococcus oeni* phage fOg44 is endowed with a bona-fide SP that is processed by the leader peptidase during infection and is exported by the *sec* machinery [13]. A survey of orthologous endolysins from other phages of Gram-positive hosts suggested that some of these have N-terminal sequences resembling secretory signals, although in every case an adjacent holin gene was also present [6,13]. In addition, the endolysin of *Lactobacillus plantarum* phage ϕ g1e has been reported as being exported in *E. coli* by the Sec machinery [14]. Particularly remarkable cases are the endolysins of *E. coli* phages P1 and 21, which feature an N-terminal signal-arrest-release (SAR) sequence that allows the enzyme to be exported to the membrane where it is arrested, and to be released as a soluble active enzyme in the periplasm [15–17].

The mycobacteriophage Ms6 is a temperate bacteriophage with an unusual lytic cassette: in addition to the endolysin-holin lysis system, encoded by genes *lysA* (*gp2*) and *gp4/gp5*, respectively, the Ms6 lytic cassette comprises two additional lysis proteins encoded by genes *gp1* and *gp3* (*lysB*) [18,19]. The *lysB* gene has been previously characterized: it encodes an enzyme with lipolytic activity that hydrolyzes the mycolic acids from the mycolyl-arabinogalactan-peptidoglycan complex [20,21] acting at a later stage of infection to facilitate lysis by compromising the integrity of the mycobacterial outer membrane linkage to the arabinogalactan-peptidoglycan layer [22]. The Ms6 *lysA* gene was shown to encode a 384 amino acid polypeptide (LysA) with significant similarity to some bacteriophage encoded lysins with *N*-acetylmuramoyl-L-alanine amidase activity [18]. Several types of cell wall hydrolases seem to be produced by mycobacteriophages. While phages Ms6 and TM4 encode enzymes with an amid-2 type domain [23], others such as D29 and Bxb1 employ hydrolases with lysozyme-like activity to bring about host cell lysis. Mycobacteriophage endolysins containing SP or SAR domains that allow secretion of the endolysin into the periplasmic space have not yet been described. Interestingly, however, our group has recently identified the product of *gp1* gene as a chaperone-like protein that specifically interacts with the N-terminal region of the Ms6 endolysin. Gp1 is involved in the endolysin translocation across the cytoplasmic membrane independently of the holin function and is required for efficient phage release [24]. During an attempt to purify LysA as a C-terminal histidine-tagged fusion product, we detected the synthesis of two proteins, rather than a single polypeptide. Further examination of *lysA* (*lysA*₃₈₄) nucleotide sequence revealed a second possible gene (*lysA*₂₄₁) in the same reading frame and preceded by a potential ribosome-binding site (RBS). Here, we report studies directed at dissecting the precise role of the *lysA*-encoded gene products during *Mycobacterium smegmatis* infection by the mycobacteriophage Ms6. In addition, the lytic activity spectrum of both proteins was also examined in both Gram-positive and Gram-negative bacteria and also in mycobacteria regarding the potential application of mycobacteriophage lysins.

Materials and Methods

Bacterial strains, phages, plasmids and culture conditions

Bacterial strains, phages and plasmids used throughout this study are listed in Table 1. *E. coli* strains were grown at 37°C, in Luria-Bertani (LB) broth or agar supplemented with 100 μ g ml⁻¹ ampicillin or 30 μ g ml⁻¹ kanamycin, when appropriate. *M. smegmatis* recombinant strains were grown at 37°C in 7H9 medium (Difco) supplemented with 0.05% Tween 80, with shaking or Middlebrook 7H10 (Difco), containing 15 μ g ml⁻¹ kanamycin.

For induced conditions 0.2% succinate and 0.2% acetamide were also added to media.

Plasmid construction

Unless otherwise indicated, DNA fragments obtained by PCR were amplified using Ms6 genomic DNA as template. DNA amplification, plasmid isolation and electrophoresis were carried out using standard techniques [25]. *E. coli* and *M. smegmatis* mc²155 cells were transformed as described previously [25,26]. Restriction enzymes and T4 DNA ligase (New England Biolabs) were used according to the supplier's recommendations. All oligonucleotides were purchased from Thermo Scientific and are listed in Table S1.

In order to construct plasmids pMJC40 and pMJC42, *lysA*₂₄₁ was amplified using primers *lysA*₂₄₁fwd-1/*lysA*-c3 or *lysA*₂₄₁fwd-2/*lysA*-c3 and the resulting DNA fragments were introduced into the BamHI/HindIII sites of vector pQE30, allowing a fusion to a hexahistidine tag at the N-terminus, or pET29b allowing a C-terminal hexahistidine tag fusion, respectively. To obtain plasmid pMJC41, the DNA fragment containing *lysA*, was amplified by PCR with primers gp2A/*lysA*-c3 and cloned into BamHI/HindIII sites of pET29b fused to a C-terminal His₆tag. Plasmid pMJC43 was constructed by PCR amplifying *lysA*₃₈₄ (*lysA*) lacking the GTG start codon with primers *lysA* Δ GTGfwd/*lysA*-c3 and cloning the DNA fragment, after restriction with XbaI/HindIII which removed the vector translational signals (RBS and start codons), in pET29b. All constructs were validated by sequencing the insert nucleotide sequence.

Construction of Ms6 mutant phages

Construction of Ms6 mutant phages was performed using the Bacteriophage Recombineering of Electroporated DNA (BRED) as described previously [24,27]. Briefly, for Ms6 *lysA* deletion, a 100 bp oligonucleotide, Pr Δ *lysA*, that has 50 bp of homology upstream and downstream of the region to be deleted was extended by PCR using two 75 bp extender primers, PrExt Δ *lysA*fwd/PrExt Δ *lysA*rv, which have 25 bp of homology to the ends of the 100-mer and add additional 50 bp of homology on either end. The final 200 bp dsDNA product was purified using MinElute PCR Purification Kit (QIAGEN) and co-electroporated with Ms6_{wt} genomic DNA into electrocompetent recombineering cells of *M. smegmatis* mc²155:pJV53. To abolish synthesis of Lysin₃₈₄ and Lysin₂₄₁ we designed two complementary 73 bp oligonucleotides (Pr*lysA*_{TGA}HindIII_{fwd}/Pr*lysA*_{TGA}HindIII_{rv}) that introduce a stop codon and a HindIII restriction site downstream of the start codon of *lysA*₃₈₄, or two complementary 86 bp oligonucleotides (Pr*lysA*_{GTG→TGG}MscI_{fwd}/Pr*lysA*_{GTG→TGG}MscI_{rv}) that modify the *lysA*₂₄₁ GTG start codon (valine) to TGG (tryptophan) and introduce an MscI restriction site, respectively. Complementary oligonucleotides were co-transformed with Ms6-LysAHis₆ genomic DNA into recombineering cells of *M. smegmatis* mc²155:pJV53. Cells were resuspended in 7H9 supplemented with 0.5% glucose and 1 mM of CaCl₂, incubated at 37°C for 2 hours (prior to lysis) and plated as top agar lawns with *M. smegmatis* mc²155. Phage plaques were picked into 100 μ l phage buffer (10 mM Tris-HCl, pH 7.5; 10 mM MgSO₄; 68.5 mM NaCl; 1 mM CaCl₂), eluted for two hours at room temperature and analysed by DADA-PCR [27] with primers Δ *lysA*DADA-PCR/*lysB*DADA-PCR to detect *lysA* deletion. To detect *lysA* TGA insertion and *lysA* GTG₁₄₄→TGG change, phage plaques were analysed by PCR with primers PrP1fwd/*lysA*180bprv or gp2A/gp2B followed by HindIII or MscI restriction, respectively. Mixed primary plaques containing both mutant and wild-type DNA were eluted as described above, and serial dilutions were plated with *M.*

Table 1. Strains, bacteriophages, plasmids used in this study.

Strains, bacteriophages or plasmids	Description	Reference or source
Bacteria		
<i>Escherichia coli</i>		
JM109	<i>recA1 endA1 gyr96 thi hsdR17 supE44 relA1 Δ(lac-proAB) [F' traD36 proAB lac⁺ZΔM15]</i>	Stratagene
BL21 (DE3)	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dmc</i> (DE3)	Novagen
<i>Mycobacterium smegmatis</i>		
mc ² 155	High-transformation-efficiency mutant of <i>M. smegmatis</i> ATCC 607	[26]
Bacteriophages		
Ms6 _{wt}	Temperate bacteriophage from <i>M. smegmatis</i>	[70]
Ms6-LysAHis ₆	His ₆ tag insertion at the 3' end of Ms6 <i>lysA</i>	[24]
Ms6 _{Δgp1} -LysAHis ₆	His ₆ tag insertion at the 3' end of Ms6 <i>lysA</i> in Ms6 _{Δgp1}	This study
Ms6-Lysin ₃₈₄ His ₆	GTG→TGG change in codon 144 of <i>lysA</i> in Ms6-LysAHis ₆	This study
Ms6-Lysin ₂₄₁ His ₆	Stop codon introduced in the <i>lysA</i> gene of Ms6-LysAHis ₆	This study
Plasmids		
pQE30	Expression vector; T5 promoter; Amp ^r	QIAGEN
pET29b(+)	Expression vector, T7 promoter; Kan ^r	Novagen
pJV53	Derivative of pLAM12 with Che9c 60 and 61 under control of the acetamidase promoter; Kan ^r	[71]
pMJC40	<i>lysA</i> ₂₄₁ Ms6 cloned into pQE30	This study
pMJC41	<i>lysA</i> ₃₈₄ Ms6 cloned into pET29b(+)	This study
pMJC42	<i>lysA</i> ₂₄₁ Ms6 cloned into pET29b(+)	This study
pMJC43	<i>lysA</i> _{384ΔGTG} cloned into pET29b(+) Δ RBS	This study

Ms6 lysis genes Accession No. AF319619.

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smegmatis. Individual secondary plaques or lysates were screened by DADA-PCR for *lysA* deletion or by PCR and restriction with the same primers referred above to identify pure mutant phages. Construction of phage Ms6_{Δgp1}-LysAHis₆ was done as described previously [24] using Ms6_{Δgp1} genomic DNA.

Lysin expression in *M. smegmatis*-infected cells

Examination of Lysin₃₈₄ and Lysin₂₄₁ synthesis in *M. smegmatis* was performed as previously described [13]. An exponentially growing culture of *M. smegmatis* mc²155 was infected with Ms6-LysAHis₆, Ms6_{Δgp1}-LysAHis₆, Ms6-Lysin₂₄₁His₆ or Ms6-Lysin₃₈₄-His₆ at an approximate multiplicity of infection (MOI) of 10 and incubated at 37°C for 30 minutes. Ten-milliliter samples were withdrawn at 30-min intervals; cells were pelleted by centrifugation and frozen at -20°C. After thawing, cells were concentrated 100-fold in phosphate-buffered saline supplemented with 20 mg of lysozyme ml⁻¹. After an incubation period at 37°C for 1 hour, 25 μ l of 5 \times SDS-PAGE sample buffer were added followed by incubation at 100°C for 5 minutes to complete cell lysis. *M. smegmatis* extracts were analysed by Western blotting and lysin immunodetection was performed using horseradish-peroxidase-conjugated anti-His monoclonal antibody (Roche).

One-step growth curves

One-step growth curve and burst-size determination were previously described [24]. The one step assays were carried out in *M. smegmatis* exponential growth cells using an MOI of 1. Cells were pelleted and resuspended in 1 ml of a phage suspension (Ms6_{wt}, Ms6-Lysin₂₄₁His₆ or Ms6-Lysin₃₈₄His₆) supplemented with 1 mM CaCl₂. The mixture was incubated 50 min at 37°C

to allow adsorption of the phages. 100 μ l of 0.4% H₂SO₄ was added to inactivate the non-adsorbed phages and the incubation continued for five min. The suspension was neutralized with 100 μ l of 0.4% NaOH and diluted 1:100 in 7H9 supplemented with 0.5% glucose and 1 mM CaCl₂. 1 ml samples were withdrawn every 30 min until reaching 300 min. 100 μ l of serial dilutions of each sample were plated with 200 μ l of *M. smegmatis* cells, on 7H10 as top agar lawns and the phage titer for each sample was determined after 24 h incubation at 37°C. Results are averages of three independent experiments.

Expression of Lysin₃₈₄ and Lysin₂₄₁ proteins in *E. coli*

E. coli BL21 (DE3):pMJC41 or *E. coli* BL21 (DE3):pMJC42 were grown in LB medium to an OD_{600 nm} of 0.6, and expression of the recombinant Lysin₃₈₄-His₆ or Lysin₂₄₁-His₆ was induced for 4 h following the addition of isopropyl β -D-1-thiogalactopyranoside to a final concentration of 1 mM. Bacterial cells were harvested by centrifugation, washed, resuspended in 50 mM Tris-HCl (pH 7.5) supplemented with a cocktail of protease inhibitors (Calbiochem), and disrupted by passage through a French pressure cell. Cell debris were removed by centrifugation, and the recombinant proteins present in the supernatant were analysed by SDS-PAGE, followed by Coomassie blue staining and Western blotting and detected as described above.

Assay of the antibacterial activity of Lysin₃₈₄ and Lysin₂₄₁

The antibacterial activity was screened using a sensitivity test [28,29] with some modifications. 100 μ l of an exponential growing culture of the test strain was plated on LB or 7H10+OADC (for mycobacteria) as top agar lawns. 20 μ l of induced *E. coli*:pMJC41

or *E. coli*:pMJC42 extracts containing Lysin₃₈₄ or Lysin₂₄₁ were spotted onto the bacterial lawn of the test strain and incubated overnight at 37°C. After overnight incubation, the presence of a clear zone was examined. *E. coli*:pET29b induced extract was used as a negative control. Activity assays were performed in triplicate. Several bacterial strains were used to test the range of antibacterial activity and were obtained from the American Type Culture Collection (ATCC) or from the Institute Pasteur Collection, Paris.

Zymogram analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) [30], and the zymogram assays were carried out as outlined by Piuri and Hatfull (2006) [31]. Briefly, 0.2% autoclaved and lyophilized *Micrococcus luteus* cells were included in 15% polyacrylamide gels for detection of bacteriolytic activity. Protein samples were heated for 3 min at 100°C in sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 5% mercaptoethanol; 20% glycerol; 0.01% bromophenol blue), and then separated on SDS-gels containing the autoclaved cells. After electrophoresis, the zymograms were washed for 30 min with distilled water at room temperature and then transferred into renaturation buffer containing 25 mM Tris-HCl (pH 7.5) and 0.1% Triton X-100 followed by further incubation for 16 h at 37°C. The zymograms were rinsed with distilled water, stained with 0.1% methylene blue in 0.01% KOH for 2 h at room temperature, and then destained with distilled water. Peptidoglycan hydrolase activity was detected as a clear zone on a dark blue background of stained peptidoglycan. Gels not containing peptidoglycan were stained with Coomassie Brilliant Blue. Lysozyme and bovine serum albumin (BSA) were used as positive and negative controls, respectively. Molecular masses were determined by comparison with prestained molecular weight standards that were electrophoresed on the same gel.

Results

Identification of two gene products from Ms6 *lysA*

The 1155 bp *lysA* gene of mycobacteriophage Ms6 starts at a GTG codon that overlaps the *gpI* TGA stop codon in a different reading frame, and is preceded in four nucleotides by a RBS sequence (5'-GGGAGCA-3') (Fig. S1). It encodes a 384 amino acid polypeptide with significant similarity to bacteriophage encoded *N*-acetyl-muramoyl-L-alanine amidases [18]. During an attempt to purify LysA as a C-terminal histidine-tagged fusion product (LysA-His₆), we detected the production of two proteins of ~27 kDa (Lysin₂₄₁) and ~43 kDa (Lysin₃₈₄), rather than a single polypeptide of 43 kDa, in *E. coli* crude extracts (Fig. 1A). We also observed that when the protein was tagged in the N-terminal domain, only the larger product (Lysin₃₈₄) reacted with the anti-His₆ antibody (data not shown). The fact that a time-dependent decrease of the 43 kDa form with a concomitant increase of the labelled 27 kDa form was not observed, rather both proteins seemed to be produced independently over the time (Fig. 1B), led us to consider that the smaller protein was not an N-terminal processed form of a larger precursor, but rather two independent translated products. Also supporting this notion, is the fact that analysis of the amino acid sequence of Ms6 LysA did not predict an amino-terminal signal sequence or a peptidase cleavage site [24]. Further examination of the *lysA* nucleotide sequence revealed a second potential gene entirely embedded in the same reading frame, preceded by a putative Shine-Dalgarno sequence (5'-TGGAGGT-3') utilized by Gram-positive bacteria and mycobacteria [32], 6 nucleotides upstream of the GTG start codon (Fig. S1), and designated *lysA*₂₄₁. Occurrence of an additional

translation event at the predicted location would be compatible with the observed molecular masses (~43 and 27 kDa) of the two proteins, considering that an additional His₆ tag was C-terminally fused to both proteins. This was experimentally confirmed by sequencing the amino-terminal region of the 27 kDa protein (Lysin₂₄₁). This protein was obtained by expressing LysA in *E. coli*, followed by isolation of the smaller lysin from a polyvinylidene difluoride membrane. The obtained N-terminal sequence, MPDEPRPD, matched the deduced sequence from residues 144 to 151. Of note is the fact that, following expression in *E. coli*, the larger product (Lysin₃₈₄) is in great excess when compared to the smaller protein (Lysin₂₄₁) and frequently Lysin₂₄₁ synthesis was not observed (Fig. 1C). To further clarify these results, *lysA* lacking its own GTG start codon was cloned into the XbaI/HindIII sites of pET29 which removes the Shine-Dalgarno sequence and the start codons of the expression vector. We were expecting that if Lysin₂₄₁ results in fact from a new translation event and is not the result of cleavage of Lysin₃₈₄, it should be synthesized from this construction. Western blotting analysis revealed the production of a single polypeptide with 27 kDa corresponding to Lysin₂₄₁ (Fig. 1D). This result unambiguously proves that Lysin₂₄₁ results from a new translation event and is independent of Lysin₃₈₄ synthesis.

To follow mycobacteriophage Ms6 LysA production in the course of *M. smegmatis* infection, infected cells were examined for lysin synthesis. *M. smegmatis* was infected with a Ms6 derivative mutant, Ms6-LysAHis₆ phage, where the 3' end of *lysA* gene was fused to a sequence coding for a hexahistidine tag, allowing the production of a LysA-His₆ tagged protein [24]. Samples were collected immediately before and every 30 minutes following infection until near the end of the Ms6 infection cycle. Protein extracts were prepared from such samples as described in materials and methods and checked for the presence of Histagged proteins by immunoblotting. Two proteins rather than a single lysin band, with 27 kDa and 43 kDa were detected, corresponding to the predicted molecular masses of Lysin₂₄₁-His₆ and Lysin₃₈₄-His₆, respectively (Fig. 2A). Both proteins were first detected at 90 minutes postinfection with mobilities indistinguishable from that exhibited by the protein forms of the *E. coli* expressed lysin. Taking into consideration that Lysin₃₈₄ interacts with Ms6 Gp1, we followed the production of both proteins in an infection assay with an Ms6 mutant lacking *gpI* [24]. In this assay, we observed a decrease in Lysin₃₈₄ although Lysin₂₄₁ amount was comparable to the levels detected for the wild-type phage (Fig. 2B). Similarly to what was observed during Ms6 infection, both endolysins were detected 90 minutes postinfection (Fig. 2A).

These results suggest that during *M. smegmatis* Ms6 infection, different products of the *lysA* gene are synthesized and result from the existence of two translational events that direct the production of a smaller (Lysin₂₄₁) and a larger (Lysin₃₈₄) endolysin rather than a processing event. In addition, synthesis and/or stability of Lysin₃₈₄ seem to be dependent on the Gp1 chaperone-like protein as already proposed [24].

Occurrence of Ms6 LysA-like proteins in mycobacteriophages

All mycobacteriophage genomes sequenced so far possess putative *lysA*-like genes that were grouped in the mycobacteriophage gene family Pham66-1, although this group of enzymes is not restricted to phages that infect mycobacteria [33]. The Lysin A (LysA) family of proteins appears to be a particularly highly diverse and interesting group of lytic enzymes composed of subgenic modules with reasonably defined boundaries [34] containing different peptidoglycan hydrolase motifs including amidases,

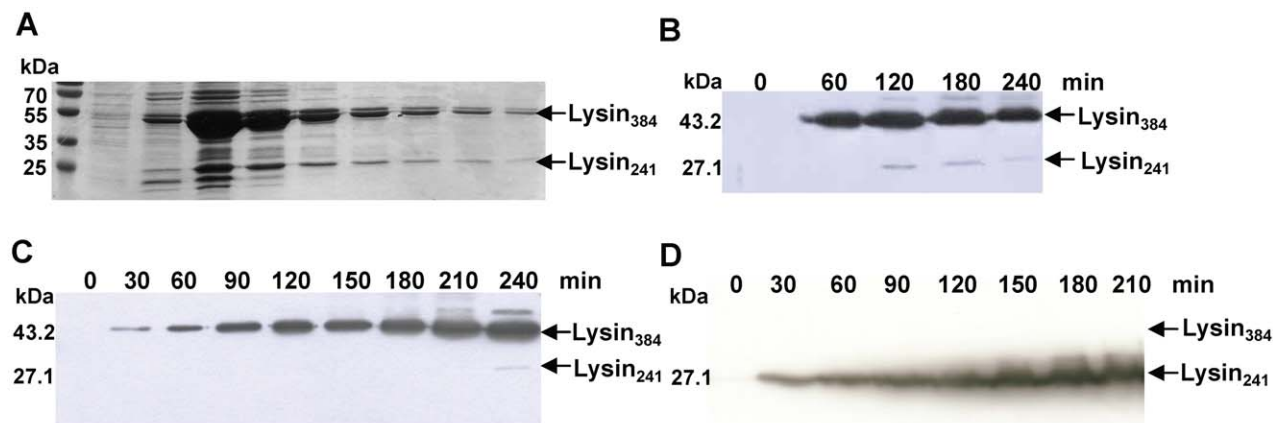


Figure 1. LysA expression in *E. coli*. **A.** Purified LysA-His₆ fractions after SDS-PAGE analysis and Coomassie Blue staining. LysA-His₆ was produced from pMJC41 in *E. coli* BL21 (DE3) after isopropyl β-D-1-thiogalactopyranoside induction. **B.** Expression of LysA-His₆ from pMJC41: detection of C-terminal His₆ tag LysA shows the production of Lysin₃₈₄-His₆ and Lysin₂₄₁-His₆. **C.** LysA-His₆ synthesis from pMJC41 over the time is not always followed by Lysin₂₄₁-His₆ production. **D.** Synthesis of Lysin₂₄₁-His₆ from pMJC43. Removal of pET29b and LysA (Lysin₃₈₄-His₆) translational signals does not hinder Lysin₂₄₁-His₆ synthesis. The molecular masses in kDa of Lysin₃₈₄ and Lysin₂₄₁ are indicated on the left; positions of both proteins are indicated by an arrow on the right. Lysin₃₈₄ and Lysin₂₄₁ were detected by Western blotting with an anti-His₆ antibody, except for panel A. doi:10.1371/journal.pone.0020515.g001

glycosidases and peptidases, as well as peptidoglycan-binding motifs [35]. Phages Ms6 and TM4 encode enzymes with an amid-2 type domain, while others such as D29 and Bxb1 employ hydrolases with lysozyme-like domains to bring about host cell lysis [23,33]. A search for conserved domains showed that Ms6 LysA holds a central peptidoglycan recognition protein (PGRP) conserved domain (cd06583), localized between amino acid residues 168 and 312 (Fig. S2). PGRPs are pattern recognition receptors that bind, and in certain cases, hydrolyze peptidoglycan of bacterial cell walls. This family includes Zn-dependent *N*-acetylmuramoyl-L-alanine amidases (EC: 3.5.1.28) which cleave the amide bond between *N*-acetylmuramoyl and L-amino acids, preferentially D-lactyl-L-Ala, in bacterial cell walls.

The mycobacteriophage Ms6 lysis module is closely related to the lysis module of phages belonging to cluster F, subcluster F1,

which includes phages PMC, Llij, Che8, Boomer, Fruitloop, Pacc40, Ramsey and Tweety [33], Ardmore [36] and Wee (GenBank accession number YP004123853). A BLASTp search for Ms6 LysA homologues identified similar proteins amongst phages of this subcluster, with a high level of identity, except for the endolysin of mycobacteriophage Pacc40, and produced significant alignments with the N-terminal region of endolysins belonging to unclustered mycobacteriophages Corndog, Phylar, Phaedrus and Pipefish and with the endolysin of phage LeBron (GenBank accession number YP003857156) [37]. Examination of the endolysin nucleotide sequences of these bacteriophages revealed the existence in all of them of a second potential translation site positioned in close proximity to the beginning of the central PGRP domain (Fig. S2, Table 2). As recently described, the accessory lysis protein Gp1 binds the N-terminal

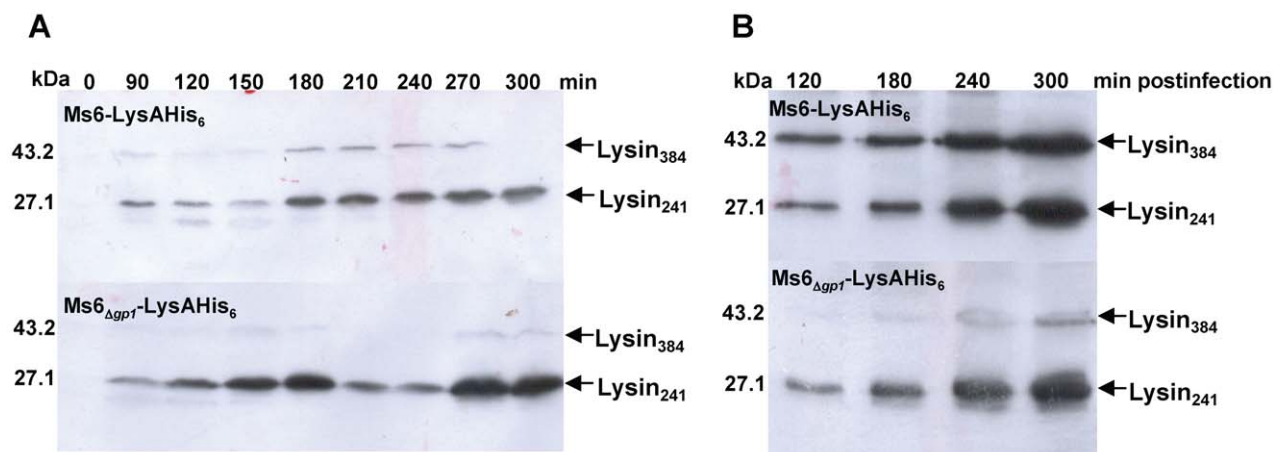


Figure 2. Time course of Lysin₃₈₄ and Lysin₂₄₁ synthesis during Ms6 infection of *M. smegmatis*. Lysin production in *M. smegmatis* was analysed after infection with Ms6-LysAHis₆ or Ms6_{Δgp1}-LysAHis₆ at an MOI of 10. Extracts were prepared from samples taken at 30-min intervals as described in Material and Methods. Samples were analysed by Western blotting and Lysin₃₈₄ and Lysin₂₄₁ synthesis was detected with an anti-His₆ monoclonal antibody. **A.** Lysin₃₈₄ and Lysin₂₄₁ synthesis is first detected 90 minutes postinfection both in Ms6-LysAHis₆ (upper panel) and Ms6_{Δgp1}-LysAHis₆ (lower panel) mutant phages. Only the results for 90 to 300 min postinfection are shown. **B.** Lysin₃₈₄ is synthesized to near undetectable levels during Ms6_{Δgp1}-LysAHis₆ infection of *M. smegmatis* (lower panel) whereas Lysin₂₄₁ production is comparable to the wild-type phage. The molecular masses in kDa of Lysin₃₈₄ and Lysin₂₄₁ are indicated on the left; positions of both proteins are indicated by an arrow on the right. doi:10.1371/journal.pone.0020515.g002

domain of LysA [24] and *gp1* was implicated in lysis because of its linkage to *lysA* and the demonstration of its requirement for mycobacteria efficient lysis [24]. *gp1*-like genes are present in many, but not all mycobacteriophage genomes and were grouped in the mycobacteriophage gene family Pham1480 [33]. In addition to subcluster F1, homologues of Ms6 Gp1 were also identified in all phages belonging to subcluster A1. However phages Lockley and Jasper did not reveal the existence of two potential translational sites in the *lysA* gene. Members of Pham1480 are mostly found adjacent to the Pham66-1 encoding genes, suggesting an even more intimate association between the gene products: while in some phages of subcluster A1 and of subcluster F1, *gp1*-like genes are overlapped with the lysin gene, in phages DD5, KBG and Solon (subcluster A1) and in phages Boomer and Ramsey (subcluster F1), Pham1480 is separated from the lysin gene by one intervening gene that code for putative homing endonuclease HNH motifs [33]. Although in these mycobacteriophages *gp1* and *lysA* are closely linked, several other mycobacter-

io-phages seem to have somewhere in their genomes genes encoding proteins that could perform functions analogous to Gp1, while others simply lack *gp1* [33].

Role of Lysin₃₈₄ and Lysin₂₄₁ during *M. smegmatis* phage infection

For a better understanding of the contribution of the endolysin to the Ms6 infection cycle, we first investigated whether its hydrolase activity is an essential function for host lysis. We used the Bacteriophage Recombineering of Electroporated DNA (BRED) strategy [27] to delete *lysA* gene (*gp2*) from the mycobacteriophage Ms6 genome. Oligonucleotides were designed to introduce a 1089 bp internal-deletion in Ms6 *lysA*, fusing 19 codons at the 5' and 3' ends of the gene to maintain the *lysB* RBS and minimize effects on expression of the adjacent *lysB* gene, as well as avoiding genetic polarity. As expected, *lysA* was essential for mycobacterial lysis and no viable phages could be recovered from a deletion of Ms6 *lysA* gene (data not shown). The same result was also reported for mycobacteriophage Giles [27].

As a result of synthesis of Lysin₂₄₁ and Lysin₃₈₄ during phage infection, we next investigated the influence of each endolysin form in phage growth parameters. Once more, we took advantage on the fact that the BRED recombineering strategy has already been described to efficiently introduce base changes that confer an amino acid substitution [27]. To eliminate synthesis of Lysin₃₈₄ or Lysin₂₄₁ we designed oligonucleotides that introduce a stop codon and a HindIII restriction site downstream of the start codon of *lysA₃₈₄*, or two complementary oligonucleotides that modify the *lysA₂₄₁* GTG start codon to TGG (tryptophan), and introduce an MscI restriction site, respectively. Both mutant phages Ms6-Lysin₃₈₄His₆ (producing Lysin₃₈₄) and Ms6-Lysin₂₄₁His₆ (producing Lysin₂₄₁) were readily isolated, demonstrating that for plaque formation only one of the two LysA forms, Lysin₃₈₄ or Lysin₂₄₁ is required (Fig. 3). Nevertheless, we considered whether the absence of one of the two endolysin forms during *M. smegmatis* phage infection (Fig. 4) could confer an altered lysis phenotype. To address this question one step growth curves and determination of phage growth parameters (latent period, rise period and burst size) were carried out to compare the phages infection cycle. The one step growth experiment (Fig. 5A) shows that in a phage Ms6-Lysin₂₄₁His₆ infection, the latent period is prolonged 30 min in comparison with the Ms6_{wt} infection and a decrease in the burst size was observed which means that a delay exists in the detection of phage release from cells infected with Ms6-Lysin₂₄₁His₆. In agreement, absence of Lysin₃₈₄ leads to smaller size phage plaques (Fig. 5B), meaning that Lysin₃₈₄ is important for infective particles release. On the other hand, Ms6-Lysin₃₈₄His₆ phage release starts 90 min later than with Ms6_{wt}. This indicates that similarly to Lysin₃₈₄, Lysin₂₄₁ has an obvious function in completion of lysis, although it does not have a significant effect in the number of phage particles released. Examination of Ms6-Lysin₃₈₄His₆ phage plaques shows that although not larger in size, plaques are more turbid than wild-type Ms6 probably due to a partial host cell lysis (Fig. 5B). These results strongly suggest that even though only one of the two LysA forms, Lysin₃₈₄ or Lysin₂₄₁, is required to accomplish host cell lysis, both enzymes are necessary for complete and efficient lysis of *M. smegmatis*.

Lysin₃₈₄ and Lysin₂₄₁ are cell wall-degrading enzymes with peptidoglycan hydrolase activity

LysA has been previously described as not affecting *E. coli* growth rate unless permeabilization of the plasma membrane by chloroform addition which results in immediate lysis [18]. To

Table 2. Occurrence of Ms6 *lysA*-like genes in mycobacteriophages.

Mycobacteriophage	<i>E. coli</i> consensus			Start Codon Position*
	SD		Start Codon	
	AGGAGGT	4-7	→ATG	
Cluster A/subcluster A1				
Bxb1	CAAGGA	tgcg	ATG	397 (1434 bp)
U2	GAAGGA	cgcg	ATG	397 (1434 bp)
Bethlehem	GAAGGA	cgcg	ATG	397 (1434 bp)
Solon	AAAAGA	tgcg	ATG	379 (1395 bp)
KBG	AAAAGA	tgcg	ATG	379 (1395 bp)
DD5	AAAAGA	tgcg	ATG	379 (1395 bp)
SkiPole	AAAAGA	ccca	ATG	397 (1413 bp)
Cluster B/subcluster B3				
Pipefish	AGGAGG	acagctccc	GTG	478 (1224 bp)
Phaedrus	AGGAGG	acagcgccc	GTG	322 (1068 bp)
Phyler	AGGAGGC	acagcgccc	GTG	520 (1266 bp)
Cluster F/subcluster F1				
Ms6	TGGAGGT	accgcc	GTG	430 (1155 bp)
Che8	CGGAGGA	acct	GTG	514 (1275 bp)
Tweety	TGGAGGT	accgcc	GTG	430 (1212 bp)
PMC	TGGAGGT	accgcc	GTG	430 (1194 bp)
Llij	TGGAGGT	accgcc	GTG	430 (1194 bp)
Boomer	CGGAGGT	tccc	ATG	523 (1302 bp)
Fruitloop	TGGAGGT	accgcc	GTG	430 (1155 bp)
Ramsey	CGGAGGA	acct	GTG	514 (1248 bp)
Pacc40	AGGAGAG	gacgcaaac	GTG	442 (1209 bp)
Wee	TGGAGGT	accgcc	GTG	430 (1155 bp)
Ardmore	TGGAGGT	accgcc	GTG	430 (1155 bp)
Unclustered				
LeBron	TGAGGT	aatc	GTG	448 (1173 bp)
Corndog	GGGAGGA	aca	GTG	439 (1221 bp)

*Numbers refer to nucleotide positions. The gene size is indicated in parenthesis.

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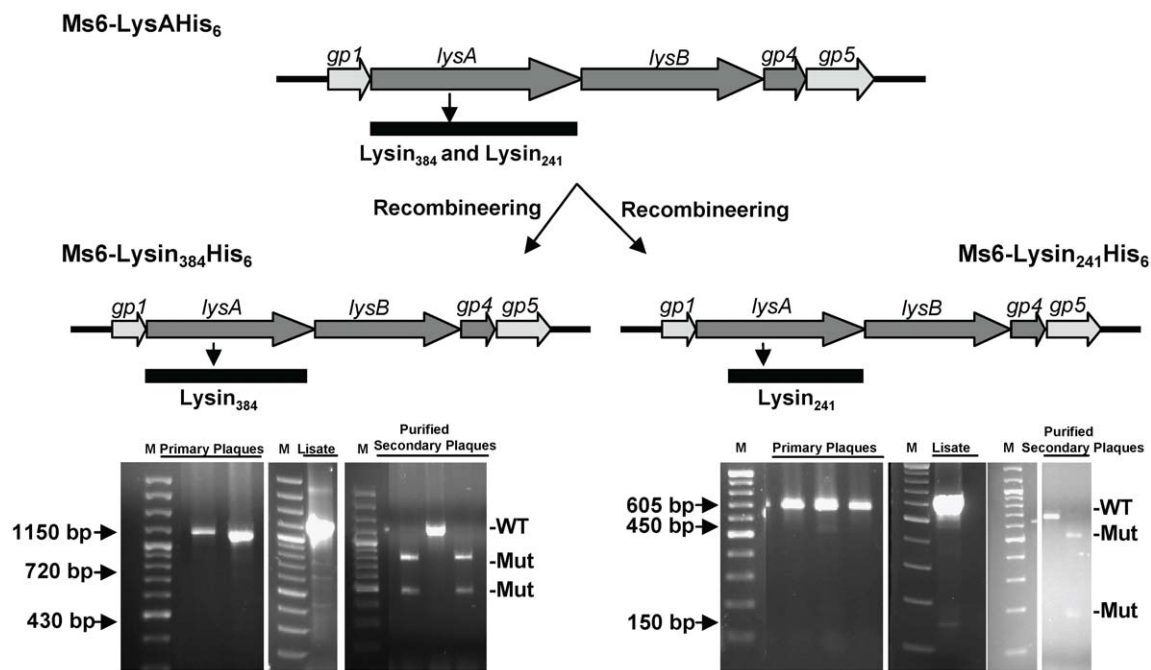


Figure 3. Construction of Ms6 *lysA* mutants. Two complementary oligonucleotides that modify *lysA*₂₄₁ GTG start codon (valine) to TGG (tryptophan) and introduce an *MscI* restriction site, or two complementary oligonucleotides that introduce a stop codon and a *HindIII* restriction site downstream of the start codon of *lysA*₃₈₄, were co-transformed with Ms6-LysAHis₆ genomic DNA; primary plaques were recovered and screened by PCR and *MscI* or *HindIII* digestion to identify a mixed plaque containing wild-type and mutant phages DNA. The mixed primary plaque was diluted and plated; the lysate was screened to check for phage viability, and purified secondary plaques were screened to identify pure mutant phages of Ms6-Lysin₃₈₄His₆ and Ms6-Lysin₂₄₁His₆, expressing only Lysin₃₈₄ or Lysin₂₄₁, respectively. doi:10.1371/journal.pone.0020515.g003

follow growth and viability of *E. coli* strains expressing Lysin₂₄₁, the *lysA* DNA fragment corresponding to *lysA*₂₄₁ was cloned into pQE30 vector allowing expression of Lysin₂₄₁ under the control of the regulated T5 bacteriophage promoter. Although induction of Lysin₂₄₁ did not result in *E. coli* lysis unless chloroform was added, growth seems to be halted over the induction period (data not shown). Ms6 LysA holds a PGRP domain and its hydrolase activity was already demonstrated; the protein was shown to cleave the bond between L-Ala and D-muramic acid and to release up to 70% of the diaminopimelic acid present in isolated mycobacterial cell walls which confirmed the amidase activity of the enzyme (unpublished results). To more directly assess the enzymatic activity of Lysin₃₈₄ and Lysin₂₄₁ we tested their ability to generate a zone of clearing in a zymogram assay. Lytic activity in lysin-producing *E. coli* extracts was checked by *in situ* protein renaturation after SDS-PAGE, using gel-incorporated autoclaved *M. luteus* cells as the substrate. As shown in Fig. 6A both Lysin₃₈₄ and Lysin₂₄₁ have hydrolase activity. Peptidoglycan hydrolytic activity in zymograms was already demonstrated for other three mycobacteriophage LysA proteins (Che8 Gp32, Bxz1 Gp236 and Corndog Gp69) [22] and TM4 Gp29 [38].

When Ms6 was first isolated, it was noticed that it forms plaques in *M. smegmatis* lawns with halos surrounding them. Formation of the halo begins after two days of incubation, once the infected area is fully formed. Halo formation around phage plaques seems to be unusual among mycobacteriophages. Nevertheless, this phenomenon has been described for, at least, mycobacteriophages Bxb1 [39] and Che12 [40] and has been observed for a number of other phages, particularly those infecting Gram-positive bacteria; plaques of phage A511 infecting *Listeria monocytogenes* were shown to form clearly visible secondary lysis zones caused by release of Ply511, a hydrophilic amidase endolysin that diffuses from the

centre of the plaque and hydrolyses the surrounding cells “from without” [7]. *M. smegmatis* cells in the exponential growth phase were infected with phages D29, Ms6_{wt}, Ms6-Lysin₂₄₁His₆ or Ms6-Lysin₃₈₄His₆. Halo formation around D29 phage plaques was not observed (Fig. 5B) while Ms6_{wt} forms turbid plaques showing size variation with partially clear halos surrounding them (Fig. 5B). When we analysed halo formation in *M. smegmatis* infected with phages Ms6-Lysin₂₄₁His₆ or Ms6-Lysin₃₈₄His₆, we observed that both phages retain the ability to form halos, although smaller and much more turbid than the wild-type phage halo (Fig. 5B). It is possible that these halos might result from the diffusion of Lysin₃₈₄ or Lysin₂₄₁ from the Ms6_{wt} phage infective centre, leading to a “lysis from without” in surrounding cells. As both endolysins forms of LysA are essential to obtain an effective lysis of *M. smegmatis*, halo turbidity could result from partial lysis of cells surrounding the phage plaques. However, these results do not rule out the existence in the Ms6 genome of other genes whose products could be implicated in the halo formation phenomenon.

Despite the fact that Ms6 LysA is produced by a phage that infects a bacterium with a complex envelope and taking into consideration the above results, we tested the activity of *E. coli* crude extracts containing Lysin₃₈₄ or Lysin₂₄₁ in mycobacteria and other bacterial species by spotting the extracts on lawns of exponential growing cells. The results, summarized in Table 3, showed that *E. coli* extracts containing Lysin₃₈₄ or Lysin₂₄₁ inhibited the growth of all mycobacteria tested except *M. fortuitum*, as well as the Gram-positive bacteria: *M. luteus*, *M. pyogenes*, *B. subtilis* and *B. pumilus*. A weak activity was observed against *S. pyogenes*, *S. aureus*, *S. epidermidis* and the Gram-negative bacterium *E. coli*. For each strain tested, a crude extract of *E. coli* BL21 (DE3):pET29b was used as a control, and no inhibition was observed for any bacterial strain (Fig. 6B). Activity on mycobac-

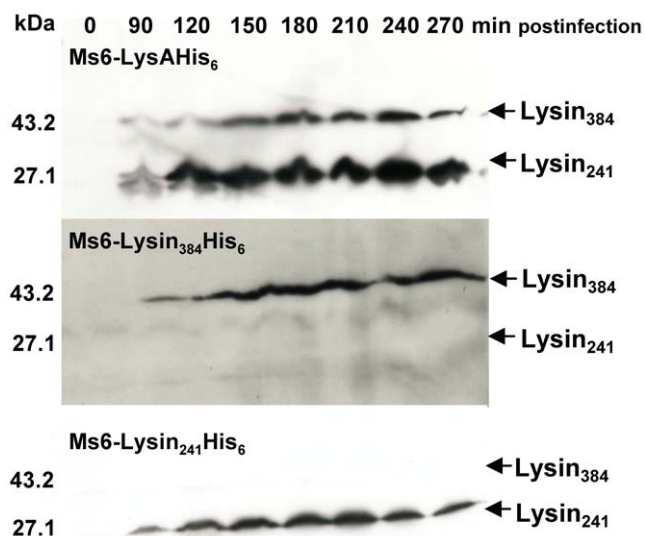


Figure 4. Time course of endolysin synthesis during Ms6-LysAHis₆, Ms6-Lysin₃₈₄His₆ or Ms6-Lysin₂₄₁His₆ infection of *M. smegmatis*. Lysin production in *M. smegmatis* was analysed after infection at an MOI of 10. Extracts were prepared from samples taken at 30-min intervals as described in Material and Methods. Samples were analysed by Western blotting and Lysin₃₈₄ and Lysin₂₄₁ synthesis was detected as already described. Both Lysin₃₈₄ and Lysin₂₄₁ synthesis could be detected beginning 90 minutes postinfection in Ms6-LysAHis₆ (upper panel). In Ms6-Lysin₃₈₄His₆ and Ms6-Lysin₂₄₁His₆, (lower panels) only Lysin₃₈₄ or Lysin₂₄₁ synthesis could be detected also beginning 90 min postinfection, respectively. Only the results for 90 to 270 min postinfection are shown. The molecular masses in kDa of Lysin₃₈₄ and Lysin₂₄₁ are indicated on the left; positions of both proteins are indicated by an arrow on the right.

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teria cells was also confirmed by the ability of bacteriophages Ms6_{wt}, Ms6-Lysin₂₄₁His₆ and Ms6-Lysin₃₈₄His₆ to form clearing zones on lawns of growing cells with *M. smegmatis* and *M. aurum* showing to be the most sensitive mycobacteria (Table 3).

Discussion

In the present work, we provide evidence that two proteins (Lysin₃₈₄ and Lysin₂₄₁) with endolysin activity are produced from the mycobacteriophage Ms6 *lysA* gene. Our group has recently described that the N-terminal domain of Lysin₃₈₄ is necessary and sufficient to directly interact with Gp1, a chaperone-like protein located upstream of LysA that assists the translocation of the endolysin across the cytoplasmic membrane in a holin-independent way [24]. Evidence for the presence of two forms of LysA raised the possibility that the shorter protein could be the mature form of Lysin₃₈₄: if a cleavage event had occurred, it would implicate the removal of the N-terminal 143 amino acids of the enzyme corresponding to 16 kDa. Although unusual, as generally mycobacterial SP length is 32 residues [41], similar to the lengths of SPs from Gram-positive bacteria [42], cleavage of a large segment of 143 amino acids that functions as a SP has already been described for the *Staphylococcus simulans* lysostaphin [43]. However, no sequence signals or cleavage sites were detected by bioinformatic analysis in the N-terminal region of LysA [24]. Examination of the *lysA* nucleotide sequence revealed the existence of a second possible lysin gene embedded within *lysA* in the same reading frame and preceded by a consensus Shine-Dalgarno sequence (Fig. S1). The existence of two translation events was

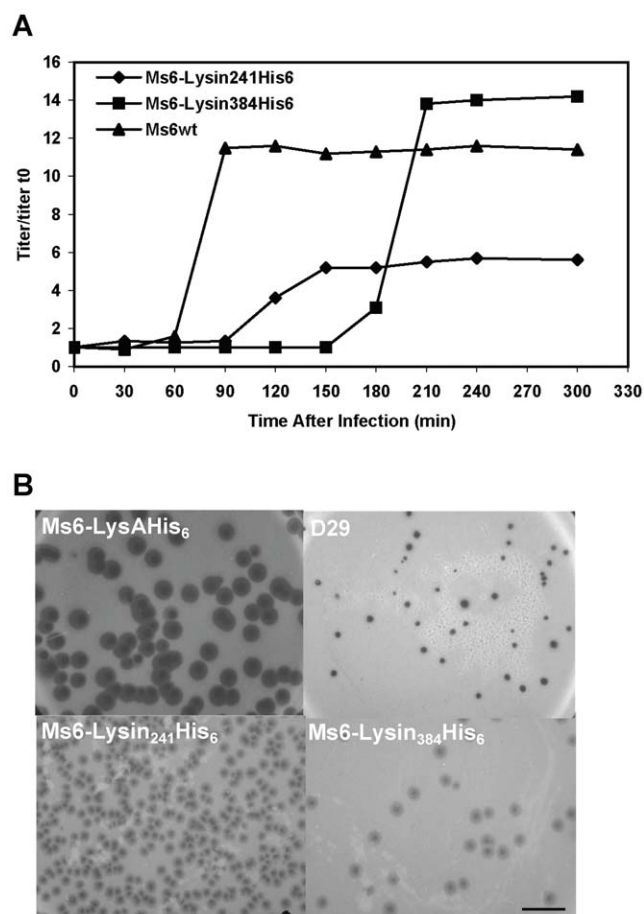


Figure 5. Both Lysin₃₈₄ and Lysin₂₄₁ are required for efficient host cell lysis. **A.** One step growth curves of Ms6 and lysin-mutant derivatives. For each curve the titers measured were divided by the titer at $t=0$ for normalization (titer/titer t_0). **B.** Halo formation by mycobacteriophage Ms6 and lysin-mutant derivatives. Serial dilutions of the bacteriophages stocks containing $\sim 10^{10}$ particles ml^{-1} were prepared and 100 μl of the 10^{-8} dilution was plated with 200 μl of an exponential growing culture of *M. smegmatis* as top agar lawns. Incubation was continued for 3 days at 37°C. Mycobacteriophage D29 was used as a negative control of halo formation [36]. Scale bar represents 1 cm.

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clearly demonstrated when Lysin₂₄₁ was still produced after elimination of the translation signals from Lysin₃₈₄, both in *E. coli* and in *M. smegmatis* phage infection: if Lysin₂₄₁ was a processed form of Lysin₃₈₄ it could not be detected. Thus, occurrence of a cleavage event, as observed for the endolysin of bacteriophage fOg44 [13] was ruled out. Coding sequences entirely encompassed within other genes seem to be very rare among dsDNA bacteriophages: λr_z and $r_z I$ represent an example of two genes located in different reading frames in the same nucleotide sequence which encode different proteins, both required in the same physiological pathway [44]. Although very rare, some exceptions among bacteriophage endolysins were found: 1) the bacteriophage $\phi vML3$ endolysin gene encodes two proteins, a larger lysin that has homology with lysozymes and a smaller lysis protein that has some features resembling those of a holin [45]; 2) the streptococcal C1 bacteriophage lysin called PlyC is a multimeric lysin composed of two separate gene products, PlyCA and PlyCB responsible for the hydrolytic amidase activity and cell-wall-binding domain, respectively [46]; 3) examination of the

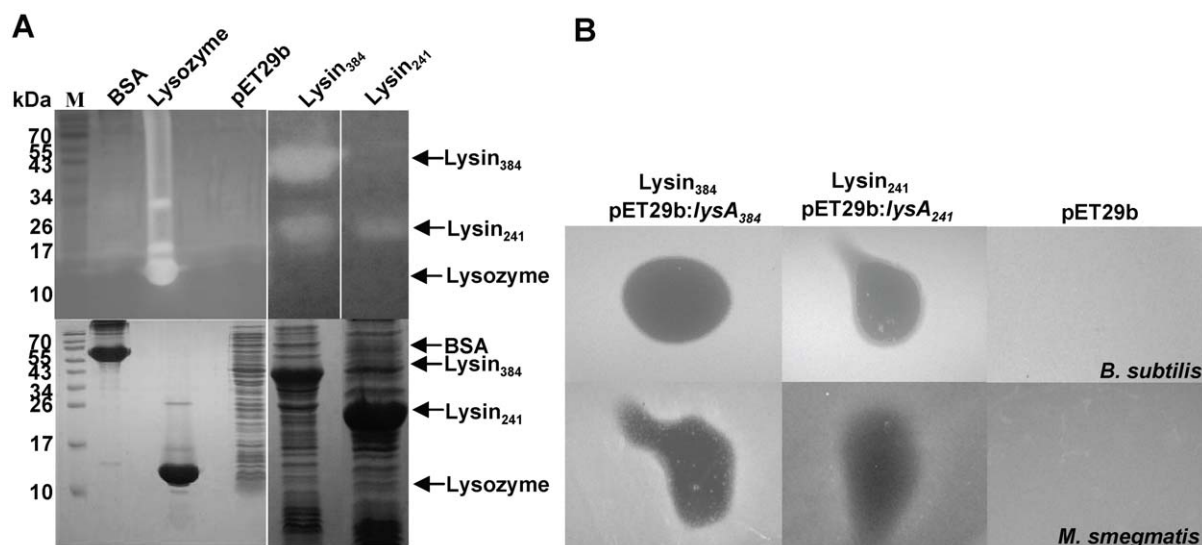


Figure 6. Peptidoglycan hydrolysis by *E. coli*-produced Lysin₃₈₄ and Lysin₂₄₁ in *M. luteus* cells. **A.** Lytic activity of lysin extracts was assessed by *in situ* renaturation after SDS-PAGE using a gel matrix containing *M. luteus* cells as substrate (upper panel). Peptidoglycan hydrolysis by renatured proteins within the gel produces clear zones that no longer stain with methylene blue. Lysozyme and bovine serum albumin (BSA) represent positive and negative controls, respectively. A cell-free control gel was run in parallel and stained with Coomassie blue (lower panel). The molecular masses in kDa of BSA, lysozyme, Lysin₃₈₄ and Lysin₂₄₁ are indicated on the left; positions of proteins are indicated by an arrow on the right. **B.** Effect of Lysin₃₈₄ or Lysin₂₄₁ activity on lawns of *B. subtilis* (upper panel) and *M. smegmatis* (lower panel). 20 μ l of *E. coli*:pMJC41 or *E. coli*:pMJC42 extracts containing Lysin₃₈₄ or Lysin₂₄₁ were spotted onto the bacterial lawn of the test strain and incubated overnight at 37°C. After overnight incubation, the presence of a clear zone was examined. *E. coli*:pET29b induced extract was used as a negative control.
doi:10.1371/journal.pone.0020515.g006

nucleotide sequence of bacteriophage CMP1 endolysin gene revealed a possible Shine-Dalgarno sequence within the gene, four nucleotides upstream of a second ATG codon in the same reading frame which would correspond to a gene product consisting of 166 C-terminal amino acid residues that includes the binding domain of the enzyme [47].

A BLASTn search for Ms6 *lysA* homologues revealed that this peculiar endolysin gene arrangement is widespread in mycobacteriophages, in particular amongst those that possess a *gp1*-like gene (Pham1480) [33], which would suggest that Gp1 may confer a selective advantage for host cell lysis under different environmental conditions: very small differences in lysis timing and

Table 3. Antibacterial activity of Ms6 and derivative mutants and its lysis proteins.

	Bacteriophage activity*			Protein activity*	
	Ms6 _w	Ms6-Lysin ₂₄₁ His ₆	Ms6-Lysin ₃₈₄ His ₆	Lysin ₃₈₄	Lysin ₂₄₁
<i>Mycobacterium smegmatis</i>	++	++	++	++	++
<i>Mycobacterium vaccae</i>	+	+	-	++	++
<i>Mycobacterium aurum</i>	++	++	-	++	++
<i>Mycobacterium fortuitum</i>	+	+	-	-	-
<i>Enterococcus faecium</i>	-	-	-	-	-
<i>Enterococcus faecalis</i>	-	-	-	-	-
<i>Streptococcus pyogenes</i>	-	-	-	+	+
<i>Micrococcus luteus</i>	-	-	-	++	++
<i>Micrococcus pyogenes</i>	-	-	-	++	++
<i>Bacillus subtilis</i>	-	-	-	++	++
<i>Bacillus pumilus</i>	-	-	-	++	++
<i>Staphylococcus aureus</i>	-	-	-	+	+
<i>Staphylococcus epidermidis</i>	-	-	-	+	+
<i>Escherichia coli</i>	-	-	-	+	+

*Designations refer to bacterial lawn clearance and are as follows: ++, clearance observable at the site of bacteriophage or lytic protein application; +, partial clearance observable at the site of bacteriophage or lytic protein application; -, no clearance.

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efficiency are strongly selective because of competition for hosts by newly released progeny [48]. However, two putative translational signals were also identified in endolysin genes belonging to five mycobacteriophages (Phyler, Phaerdrus, Pipefish, Corndog and LeBron) that do not possess Gp1 homologues but possess related Ms6 LysA sequences. The lack of representation of Pham1480 upstream of *lysA* in these phages could result from loss of *gp1*-like gene in these genomes. Furthermore, in three mycobacteriophages (TM4, Jasper and Lockley) that possess Gp1 similar proteins but unrelated Ms6 LysA enzyme, this *lysA* gene arrangement was not observed which suggests that Pham1480 in these mycobacteriophages might result from recent acquisition by horizontal genetic exchange [33]. These data also support the idea that all of these genomes have been in genetic communication, as Pham1480 is restricted to mycobacteriophages [33], and reflect the highly sequence diversity and modular nature of mycobacteriophage genomes that are characteristically mosaic comprising modules (frequently containing just a single gene) or cassettes, many of which shared by other phage genomes [49,50]. Although we have clearly shown that Ms6 produces two endolysins and that both are required for lysis, a question remains to be answered: why mycobacteriophages need to produce two endolysins, or is this phenomenon only a consequence of gene transfer through evolution? More studies with other mycobacteriophages will certainly help to clarify the need for two endolysins and for Gp1 homologues.

Although a clear relationship between Gp1 and LysA does not seem to occur among all mycobacteriophages, our results indicate that in Ms6 a tight association between the two proteins exists. We observed that synthesis and/or stability of the larger endolysin (Lysin₃₈₄) is highly dependent on Gp1 production. A reasonable explanation is that in the absence of its chaperone, the endolysin becomes unstable in the cytoplasm, or that an efficient translation of LysA is more or less dependent upon translation of the adjacent *gp1*-coding region as suggested by overlapping stop/start codons: Lysin₃₈₄ expression may rely on the ribosome frameshifting at the GTGA sequence joining the *gp1* and *lysA* reading frames. When analysing LysA production during the infective cycle of both Ms6-LysAHis₆ and Ms6 Δ_{gp1} -LysAHis₆ phages, we observed a decrease in the Lysin₃₈₄ levels in cells infected with Ms6 Δ_{gp1} , although Lysin₂₄₁ synthesis remains apparently unaffected. However, this is not the result of a polar effect at the transcriptional level as infection of *M. smegmatis* cells expressing the wild-type Gp1 protein in trans, with Ms6 Δ_{gp1} , leads to a reversion of the lysis defect [24]. Construction of Ms6 mutant phages deleted in *lysA* or defective for Lysin₃₈₄ or Lysin₂₄₁ synthesis showed that LysA is essential for host cell lysis. In fact, as pointed out by R. Young (2005) [48] endolysins are always essential (for dsDNA phages) in terms of plaque-forming ability, whereas holins may be not; indeed, for mycobacteriophage Ms6, LysA is the only lysis function that can not be suppressed and is indispensable for lysis, even though deletion of the additional lysis genes (*gp1*, *lysB*, *gp4* and *gp5*) may result in poor phage viability and severe lysis defects [19,24; Catalão and Gil, unpublished results]. Suppression of Lysin₃₈₄ or Lysin₂₄₁ synthesis does not result in a non-lysis phenotype as both proteins harbour the PGRP domain. However, lack of Lysin₃₈₄ or Lysin₂₄₁ in phage virion results in an altered lysis phenotype; analysis of the Ms6-Lysin₂₄₁His₆ (defective for Lysin₃₈₄ synthesis) and Ms6-Lysin₃₈₄His₆ (defective for Lysin₂₄₁ synthesis) phage growth parameters revealed that, whereas Lysin₃₈₄ is necessary to achieve a normal burst of infective phages, Lysin₂₄₁ has an important function in the progression and complete host cell lysis. At this time it is unknown if Lysin₃₈₄ activation is dependent on holin function. It is possible that Gp1 plays a role in maintenance

of Lysin₃₈₄ inactive state: Gp1 binding to the N-terminal domain may alter the endolysin conformation and block substrate binding or Gp1 may allow Lysin₃₈₄ to adopt an active conformation. Indeed, the fact that Lysin₃₈₄ is detected almost exclusively in the presence of Gp1 suggests that Gp1 might affect the stability of the endolysin. Chaperone-synthesis/stability dependence has been already described for some lipases [51–54]. The energized state of the cytoplasmic membrane was also described as being implicated both in autolysins activation [55–58] and secretory endolysins activation [13,15,59].

Remarkably, *E. coli* extracts containing Lysin₃₈₄ or Lysin₂₄₁ enzymes inhibited bacterial growth of most of the Gram-positive bacteria and mycobacteria tested (that included *M. smegmatis*, *M. vaccae*, *M. aurum* and *M. fortuitum*) contrary to what was previously thought [22,36]. This data is in agreement with the ability of Ms6 to form turbid plaques surrounded by a clear zone of apparent bacterial growth inhibition. This phenomenon is widely observed among bacteriophages that infect Gram-positive hosts and results from “lysis from without” of bacteria as a result of endolysin diffusion from phage plaques that kills uninfected cells [7]. Although *Mycobacterium* spp. are Gram-positive bacteria included in the suborder of *Corynebacterineae*, the envelope of this bacterial group is composed of a typical plasma membrane surrounded by a cell wall core, which, in turn, is surrounded by an outer membrane layer [60,61].

Unexpectedly, we observed that different mycobacterial species are susceptible to exogenously added Lysin₃₈₄ and Lysin₂₄₁, despite their mycolic-acid-rich outer membrane. Even though it is unlikely that Lysin₃₈₄ or Lysin₂₄₁ can diffuse through water-filled channels, the porins [62,63], as typically only molecules with masses up to 600 Da can pass through the pores [64], it is possible that lysin access to the peptidoglycan may occur during cell division and septal peptidoglycan biogenesis. This is of interest as exogenously applied phage-encoded endolysins have been shown to possess effective antimicrobial activity [65] against Gram-positive bacterial pathogens [66–69]. The ultimate challenge will be engineer improved mycobacteriophage lysins with higher activity and test the synergistic effect with other enzymes as LysB or outer membrane permeabilizers that could facilitate the access of LysA to the peptidoglycan.

Supporting Information

Figure S1 Relevant features of the DNA sequence including and surrounding the Ms6 *lysA* gene. Putative RBS consensus sequences from *lysA*₃₈₄ and *lysA*₂₄₁ are shown in bold and underlined. Translational start and stop codons are superscripted and/or in bold. Amino acids residues of LysA are indicated below the nucleotide sequence. Amino acid substitutions and insertions to construct Ms6 *lysA* mutant phages are highlighted; Ms6-Lysin₂₄₁His₆ has a stop codon and a HindIII restriction site, downstream of the *lysA* start codon which eliminates synthesis of Lysin₃₈₄; substitution of the GTG codon by TGG at position 144 eliminates synthesis of Lysin₂₄₁ (Ms6-Lysin₃₈₄His₆); Ms6-LysAHis₆ [23] and Ms6 Δ_{gp1} -LysAHis₆ have a five histidine insertion just before the TGA stop codon to generate a His₆tag C-terminal fusion with *lysA*. (TIF)

Figure S2 CLUSTALW alignment of Ms6 LysA and putative LysA amino acid sequences of subcluster F1 mycobacteriophages. Mycobacteriophages: Llij Gp30 (YP655026), PMC Gp30 (YP655791), Ms6 Gp2 (AAG48318), Fruitloop Gp29 (YP002241714), Ardmore Gp29 (YP003495170), Tweety Gp30 (YP001469263), Wee Gp31 (YP004123853), Che8

Gp32 (NP817370), Boomer Gp32 (YP002014248) and Ramsey Gp32 (YP002241819); the primary accession numbers of the UniProtKB/TrEMBL database are given in parenthesis. Identical (*), highly similar (:) and similar (.) amino acids are indicated. Dashes represent gaps introduced by CLUSTALW to optimize the alignment. The PGRP conserved domain is highlighted on a grey background. Numbers refer to amino acid positions. Predicted start codons are shown in bold. (TIFF)

Table S1 Oligonucleotides used in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: MJC JM-P MP. Performed the experiments: MJC CM FG. Analyzed the data: MJC CM FG JM-P MP. Wrote the paper: MJC MP.

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