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Functional Analysis of the Holin-Like Proteins of Mycobacteriophage Ms6▽†

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The mycobacteriophage Ms6 is a temperate double-stranded DNA (dsDNA) bacteriophage which, in addition to the predicted endolysin (LysA)-holin (Gp4) lysis system, encodes three additional proteins within its lysis module: Gp1, LysB, and Gp5. Ms6 Gp4 was previously described as a class II holin-like protein. By analysis of the amino acid sequence of Gp4, an N-terminal signal-arrest-release (SAR) domain was identified, followed by a typical transmembrane domain (TMD), features which have previously been observed for pinholins. A second putative holin gene (*gp5*) encoding a protein with a predicted single TMD at the N-terminal region was identified at the end of the Ms6 lytic operon. Neither the putative class II holin nor the single TMD polypeptide could trigger lysis in pairwise combinations with the endolysin LysA in *Escherichia coli*. One-step growth curves and single-burst-size experiments of different Ms6 derivatives with deletions in different regions of the lysis operon demonstrated that the gene products of *gp4* and *gp5*, although nonessential for phage viability, appear to play a role in controlling the timing of lysis: an Ms6 mutant with a deletion of *gp4* (Ms6$_{\Delta gp4}$) caused slightly accelerated lysis, whereas an Ms6$_{\Delta gp5}$ deletion mutant delayed lysis, which is consistent with holin function. Additionally, cross-linking experiments showed that Ms6 Gp4 and Gp5 oligomerize and that both proteins interact. Our results suggest that in Ms6 infection, the correct and programmed timing of lysis is achieved by the combined action of Gp4 and Gp5.

The majority of double-stranded DNA (dsDNA) bacteriophages described to date terminate each infection cycle through the programmed and regulated activity of two phage-encoded proteins, the endolysin and the holin, a small membrane protein that controls the endolysin function and the access to the peptidoglycan (39, 40). Endolysins are characterized by their ability to directly target covalent bonds in the peptidoglycan layer of the bacterial cell wall; the result of this activity is disruption of the rigid murein layer and release of newly synthesized virions (17, 42). During phage assembly, holin molecules accumulate in the cytoplasmic membrane without a detectable effect on the host (11, 37). Then, at an allele-specific time programmed into their primary structure, holins trigger to disrupt the cytoplasmic membrane (8, 11). Holins are extremely diverse and are found with at least three membrane topologies in many unrelated sequence families, suggesting that they may have evolved from multiple distinct origins to allow precisely scheduled efficient lysis and rapid adjustment of the lysis time, either on the basis of genetic selection or, in some cases, in real time in response to environmental changes (37, 41). The canonical holins, such as those of phages λ and T4, form very large holes that allow fully folded and active endolysins accumulated in the cytosol to pass through the cytoplasmic membrane and attack the peptidoglycan. These holes are nonspecific and allow the passage of unrelated endolysins (41) and proteins larger than 480 kDa (38). In addition, hole formation is absolutely required for lysis. Many phages also encode an antiholin, which contributes to control the timing of host lysis by inhibiting the holin. In some cases, the antiholin is encoded by the holin gene, with an additional N-terminal extension of several amino acids—a dual-start motif (1)—or is translated from an alternative intragenic start codon (7, 35). In other cases, the antiholin is encoded by an independent gene (27, 36, 44).

Recently, an alternative and remarkably different class of holin-endolysin systems became known (22, 23). This class, represented by the lambdoid bacteriophage 21, utilizes endolysins having N-terminal secretory signal-arrest-release (SAR) signals and pinholins. For phages encoding SAR endolysins, the holin protein needs only to produce lesions large enough to allow the passage of ions and depolarize the cytoplasmic membrane in order to fulfill its role in controlling the timing of lysis. Indeed, unlike lesions formed by the λ holin, lesions formed by the phage 21 holin do not allow the passage of λ endolysin (23). The term "pinholin" has been proposed to differentiate the small-hole (pinhole)-forming character of the phage 21 holin from the canonical holins that form large, nonspecific holes (23).

The genetic organization of the mycobacteriophage Ms6 lysis functions was previously described (4). In addition to the endolysin (*lysA*) and a holin-like gene (*gp4*), three accessory lysis genes restricted to mycobacteriophages were also identified: *gp1*, *lysB*, and *gp5*. The *gp1* gene was recently identified as encoding a chaperone-like protein that specifically interacts with the N-terminal region of LysA and is involved in its delivery to the peptidoglycan in a holin-independent manner (2). The Ms6 holin-like protein, encoded by *gp4* (previously named *hol*), shares some structural characteristics with class II holins, which are usually hydrophobic in nature and small, with a hydrophilic carboxy-terminal domain and two potential

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TABLE 1. Strains, bacteriophages, and plasmids used in this study[a]

Strain, bacteriophage, or plasmid	Description	Reference or source
Strains		
E. coli		
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	Stratagene
BL21	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm*	Novagen
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
M. smegmatis		31
mc^{2}155	High-transformation-efficiency mutant of *M. smegmatis* ATCC 607	
Bacteriophages		
D29	Lytic phage that infects both fast- and slow-growing mycobacterial species	Institute Pasteur collection
λgt11	*c*Its857 Sam100	Stratagene
Ms6$_{wt}$	Temperate bacteriophage from *M. smegmatis*	26
Ms6$_{\Delta gp1}$	213-bp in-frame deletion of the Ms6 *gp1* gene	2
Ms6$_{\Delta gp4}$	210-bp in-frame deletion of the Ms6 *gp4* gene	This study
Ms6$_{\Delta gp5}$	366-bp in-frame deletion of the Ms6 *gp5* gene	This study
Ms6$_{\Delta gp1\ gp4}$	213-bp and 210-bp in-frame deletions of the Ms6 *gp1*and *gp4* genes, respectively	This study
Ms6$_{\Delta gp1\ gp5}$	213-bp and 366-bp in-frame deletions of the Ms6 *gp1*and *gp5* genes, respectively	This study
Plasmids		
pQE30	Expression vector, T5 promoter; Amp^r *lacI*^{q}	Qiagen
pET29a(+)	Expression vector, T7 promoter; Kan^r	Novagen
pJV53	Derivative of pLAM12 with Che9c *60* and *61* under control of the acetamidase promoter; Kan^r	34
pMG231A	*lysA* cloned into pQE30	4
pMP300	Ms6 *lysA* and *gp4* cloned in pQE30	4
pMP310	Ms6 *gp4* cloned in pQE30	4
pMJC21	Ms6 *gp5* cloned in pQE30	This study
pMJC22	Ms6 *gp4* and *gp5* cloned in pQE30	This study
pMJC23	D29 *gp11* cloned in pQE30	This study
pMJC24	λ*R* and Ms6 *gp4* cloned in pQE30	This study
pMJC25	λ*R* and Ms6 *gp5* cloned in pQE30	This study
pMJC27	Ms6 *gp5* cloned in pMG231A	This study
pMJC28	Ms6 *gp4* and *gp5* cloned in pMG231A	This study
pMJC29	D29 *gp11* cloned in pMG231A	This study
pMJC30	Ms6 *gp4* cloned in pET29a(+)	This study
pMJC31	Ms6 *gp5* cloned in pET29a(+)	This study
pMJC32	Ms6 *gp4* and *gp5* cloned in pET29a(+)	This study

[a] The accession number for the Ms6 lysis genes is AF319619.

transmembrane domains (TMD1 and TMD2). Gp4 holin function was also supported by its ability to complement a λ phage S mutant (suggesting that the Ms6 holin allows the nonspecific release of the λR endolysin to the periplasm) and by the lethal phenotype observed when Gp4 is overexpressed in *Escherichia coli*, which is explained by the introduction of nonspecific lesions in the cytoplasmic membrane. However, unlike some holins, such as λ S, Gp4 lacks a dual-start motif (4). In addition, a second putative holin-like gene (*gp5*) encoding a protein with a predicted single TMD at the N-terminal region was identified at the end of the Ms6 lytic operon. In this work we studied the function of *gp4* and *gp5* gene products and demonstrated that, although nonessential for the Ms6 infective cycle, both appear to play a role in controlling the timing of lysis in mycobacteria. We also present evidence that the Ms6 holin-like protein encoded by *gp4* (here, designated Gp4 to avoid confusion) has characteristic features of a pinholin and that Gp5 encodes a holin-like protein, and we suggest that the combined action of these two proteins is essential to effect host cell lysis at the correctly timed programmed lysis.

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 μg ml^{-1} ampicillin or 30 μg ml^{-1} kanamycin, when appropriate. *Mycobacterium smegmatis* recombinant strains were grown at 37°C in 7H9 medium (Difco) supplemented with 0.05% Tween 80 and 0.5% glucose, with shaking, or in Middlebrook 7H10 (Difco) medium containing 15 μg ml^{-1} kanamycin. For induced conditions, 0.2% succinate and 0.2% acetamide were also added to media.

Plasmid construction. Unless indicated otherwise, the DNA fragments were obtained by PCR using Ms6 genomic DNA as a template. DNA amplification, plasmid isolation, and electrophoresis were carried out using standard techniques (28). *E. coli* and *M. smegmatis* mc^{2}155 cells were transformed as described previously (28, 31). Restriction enzymes and T4 DNA ligase (New England BioLabs) were used according to the supplier's recommendations. All oligonucleotides were from Thermo Scientific and are listed in Table S1 in the supplemental material.

In order to construct plasmids pMJC21 and pMJC22, DNA fragments containing the *gp5* or *gp4* and *gp5* genes were obtained by PCR amplification with primers Porf5a/Porf5-c2 or Porf4-1/Porf5-c2, respectively. Primers were designed in order to generate restriction sites, and the DNA fragments were inserted in the same sites of vector pQE30 (Qiagen), allowing fusion to a hexahistidine tag at the N terminus. To obtain plasmid pMJC23, the DNA fragment containing the

gene *gp11* was amplified by PCR using D29 genomic DNA as a template with primers P*hol*D29fwd/P*hol*D29rv and cloned into SacI/HindIII sites of pQE30. pMJC24 and pMJC25 were constructed in two steps: the λ*R* gene was amplified using the genomic DNA of bacteriophage λgt11 as a template with primers Pλ*R*fwd/Pλ*R*rv and cloned into BamHI/SacI sites of pQE30. The *gp4* or *gp5* gene was amplified by PCR using Ms6 genomic DNA as a template with primers Porf4-1/Porf4-c1 or *gp5*RBSfwd/Porf5-c2 and cloned into SacI/HindIII sites of pQE30:λ*R*, generating plasmids pMJC24 and pMJC25, respectively. pMJC27, pMJC28, and pMJC29 were obtained by amplifying *gp5* or *gp4* and *gp5* with primers *gp5*RBSfwd/Porf5-c2 or Porf4-1/Porf5-c2 or D29 *gp11* with primers P*hol*D29fwd/P*hol*D29rv using the genomic DNA of mycobacteriophage D29 as a template. The DNA fragments were introduced into the SacI/HindIII sites of pMG231A. To obtain plasmids pMJC30, pMJC31, and pMJC32, the *gp4*, *gp5*, or *gp4* and *gp5* genes of Ms6 were amplified with primers Pr*gp4*Ms6fwd/*gp4*Ms6rv, *gp5*Ms6fwd/*gp5*Ms6rv, or Pr*gp4*Ms6fwd/*gp5*Ms6rv, respectively, and cloned into BamHI/HindIII sites of pET29a(+). All constructs were validated by verifying the nucleotide sequence of the insert.

Protein interaction experiments: cross-linking. Bis (sulfosuccinimidyl) suberate (BS^3) cross-linker stock solution at a 10 mM final concentration was prepared immediately before use to decrease the extent of hydrolysis in 20 mM Na-HEPES–200 mM NaCl, pH 7.0. For *in vitro* cross-linking experiments, *E. coli* BL21(DE3) carrying plasmid pMJC30, pMJC31, or pMJC32 was induced at the logarithmic growth phase with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and 10-ml samples were withdrawn and pelleted after 1 h. Cells were resuspended in phosphate-buffered saline (PBS), broken by sonication, and centrifuged at 4°C. The proteins of the membrane fraction were extracted with 1% Triton X-100 for 2 h at 37°C. The detergent fraction was treated with BS^3 solution to a final concentration between 1 and 5 mM at room temperature for 30 min. In the control samples, the cross-linker was omitted. After incubation at room temperature, samples were resuspended in SDS-PAGE sample buffer that quenches the reaction. Aliquots were subjected to SDS-PAGE, and Gp4 or Gp5 proteins were detected by Western blotting using horseradish peroxidase (HRP)-conjugated anti-His_6 monoclonal antibody (Roche).

Beta-galactosidase activity assay. β-Galactosidase activity (20) was measured in the supernatants of induced *E. coli* BL21 cells carrying plasmids pQE30, pMP310, pMJC21, pMJC22, and pMJC23. Following a 1-h induction with 1 mM IPTG, 1-ml aliquots of exponential growing cultures were centrifuged, and 30 μl of supernatant was added to 66 μl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution (Sigma) (4 mg/ml in 0.1 M sodium phosphate buffer, pH 7.5), 3 μl of 4.5 M β-mercaptoethanol, 0.1 M $MgCl_2$ solution, and 200 μl of 0.1 M sodium phosphate buffer, pH 7.5. The reaction was performed at 37°C for 30 min and then stopped by the addition of 500 μl of Na_2CO_3. The amount of *o*-nitrophenol released was measured at 405 nm. Enzyme activity was expressed in arbitrary units of the optical density at 405 nm (OD_{405}) ml^{-1} of culture min^{-1}.

Construction of Ms6 mutants. Construction of Ms6 mutants was performed using bacteriophage recombineering of electroporated DNA (BRED) in *M. smegmatis*. Recombineering substrates and BRED strategy were done as described previously (2, 19). Briefly, for deletion of the Ms6 *gp4*, *gp5*, or *gp4* and *gp5* genes (yielding $Ms6_{\Delta gp4}$, $Ms6_{\Delta gp5}$, or $Ms6_{\Delta gp4\ gp5}$, respectively), 100-bp oligonucleotides, PrΔ*gp4*, PrΔ*gp5*, or PrΔ*gp4gp5*, that have 50 bp of homology upstream and downstream of the region to be deleted, were extended by PCR using two 75-bp extender primers, PrExtΔ*gp4*fwd/PrExtΔ*gp4*rv, PrExtΔ*gp5*fwd/PrExtΔ*gp5*rv, or PrExtΔ*gp4*fwd/PrExtΔ*gp5*rv, respectively, which have 25 bp of homology to the ends of the 100-mer and add an additional 50 bp of homology on either end. For deletion of the Ms6 *gp1* gene, a 100-bp oligonucleotide (PrΔ*gp1*) was extended with the primers PrExtΔ*gp1*fwd/PrExtΔ*gp1*rv. The final 200-bp dsDNA products were purified using a MinElute PCR purification kit (Qiagen) and coelectroporated with Ms6 genomic DNA (for *gp4*, *gp5*, or *gp4* and *gp5* deletion) or with $Ms6_{\Delta gp4}$ or $Ms6_{\Delta gp5}$ genomic DNA (for *gp1* deletion) into electrocompetent recombineering cells of *M. smegmatis* mc^2155/pJV53. Cells were resuspended in 7H9 medium supplemented with 0.5% glucose and 1 mM $CaCl_2$, incubated at 37°C for 2 h, and plated as top agar lawns with *M. smegmatis* mc^2155. Phage plaques were picked into 100 μl of phage buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$, 68.5 mM NaCl, 1 mM $CaCl_2$), eluted for 2 h at room temperature, and analyzed by PCR with primers PrP1Fwd/Pr*lysA*180bprv flanking the *gp1* deletion or with primers *lysB*fwd/Ms6rv to detect the *gp4*, *gp5*, or *gp4* and *gp5* deletions. Mixed primary plaques containing both the deletion mutant and the wild-type (wt) DNA were eluted as described above, and serial dilutions were plated with *M. smegmatis.* Individual secondary plaques or lysates were screened by PCR with primers flanking the deletions for the presence of pure mutant phage.

One-step growth curves and burst size determination. One-step growth curve and burst size determination were described previously (2). The one-step assays were carried out in cells in exponential growth using a multiplicity of infection (MOI) of 1. *M. smegmatis* cells were pelleted and resuspended in 1 ml of a phage suspension (wt Ms6 [$Ms6_{wt}$], $Ms6_{\Delta gp1}$, $Ms6_{\Delta gp4}$, $Ms6_{\Delta gp5}$, $Ms6_{\Delta gp1\ gp4}$ or $Ms6_{\Delta gp1\ gp5}$) supplemented with 1 mM $CaCl_2$. The mixture was incubated for 50 min at 37°C to allow adsorption of the phage. One hundred microliters of 0.4% H_2SO_4 was added to inactivate the nonadsorbed phage, and the incubation was continued for five min. The suspension was neutralized with 100 μl of 0.4% NaOH and diluted 1:100 in 7H9 medium supplemented with 0.5% glucose and 1 mM $CaCl_2$. One-milliliter samples were withdrawn every 30 min up to 300 min. One hundred microliters of serial dilutions of each sample was plated with 200 μl of *M. smegmatis* cells on 7H10 medium as top agar lawns, and the phage titer for each sample was determined after 24 h of incubation at 37°C. The same experimental procedure was used for burst size determination except that 10 μl of infected cells was diluted in order to obtain one infected cell ml^{-1} in 7H9 medium. Samples of 1 ml of infected cultures were distributed in 50 tubes and incubated for 180 min at 37°C. A total of 200 μl of *M. smegmatis* cells and top agar (4 ml) were added to each tube and plated on 7H10 medium. After 24 h at 37°C, the phage plaques were counted, and the Poisson distribution of [$P(n)$] was applied to determine the burst size (BS): $P(n) = (e^{-c} \cdot c^n)/n!$ ($e < 1$), where $P(n)$ is the probability of samples having n infected cells, c is the average number of infected cells per tube, and BS = (total plaque count in the 50 plates)/(total number of infected cells).

RESULTS

Sequence analysis of the Ms6 holin-like genes. The mycobacteriophage Ms6 *gp4* encodes a 77-amino-acid polypeptide with a predicted molecular mass of 7.8 kDa that was previously described as a holin-like protein (4). This assumption was based on several features of Gp4: its high similarity with the *Lactococcus lactis* bacteriophage r1t holin, a deduced amino acid sequence sharing characteristics with the class II holins, its high toxicity when overexpressed in *E. coli* leading to a lethal phenotype, and, finally, the ability to complement a lambda S mutant. However, unlike other class II holins, such as the S holin of bacteriophage 21, Ms6 Gp4 lacks a dual-start motif (4). The availability of more than 60 mycobacteriophage sequenced genomes has placed the Ms6 holin in the gene phamily Pham 95, according to sequence similarity to putative holin genes from mycobacteriophages of subcluster F1 (Fig. 1A) (12). Recently, we have reported that export of the Ms6 endolysin (LysA) is holin independent and that LysA translocation across the cytoplasmic membrane is assisted by Gp1, a chaperone-like protein, encoded by the first gene of the Ms6 lysis cassette (2). These data, together with the absence of a lysis phenotype when Gp4 was coexpressed in *E. coli* with Ms6 endolysin (4), even when both proteins were shown to be expressed at detectable levels (M. J. Catalão, unpublished data), led us to reanalyze the amino acid sequence of Ms6 Gp4.

The Ms6 Gp4 possesses two TMDs, the most hydrophobic of which is TMD2 (residues 39 to 58) (Fig. 1B) and a predicted N-in, C-in topology according to the HMMTOP (http://www.enzim.hu/hmmtop/) program from the Expasy server. TMD1, not predicted by every TMD search algorithm, is present from residues 17 to 34 and has characteristics of a SAR domain, with a high percentage (11 out of 18) of weakly hydrophobic or polar uncharged residues (Fig. 1B), like Ala, Gly, Gln, and Thr (22). The presence of a SAR domain followed by a typical TMD suggests that Ms6 Gp4 is a pinholin, analogous to other pinholins already characterized, such as the holin of phage 21, $S^{21}68$ (21, 23), or the holin of phage Xfas53 (32). As already mentioned, the Ms6 lysis module is organized into five genes, and, so far, no function has been assigned to the last gene within the lysis cassette. *gp5* has the potential to encode a

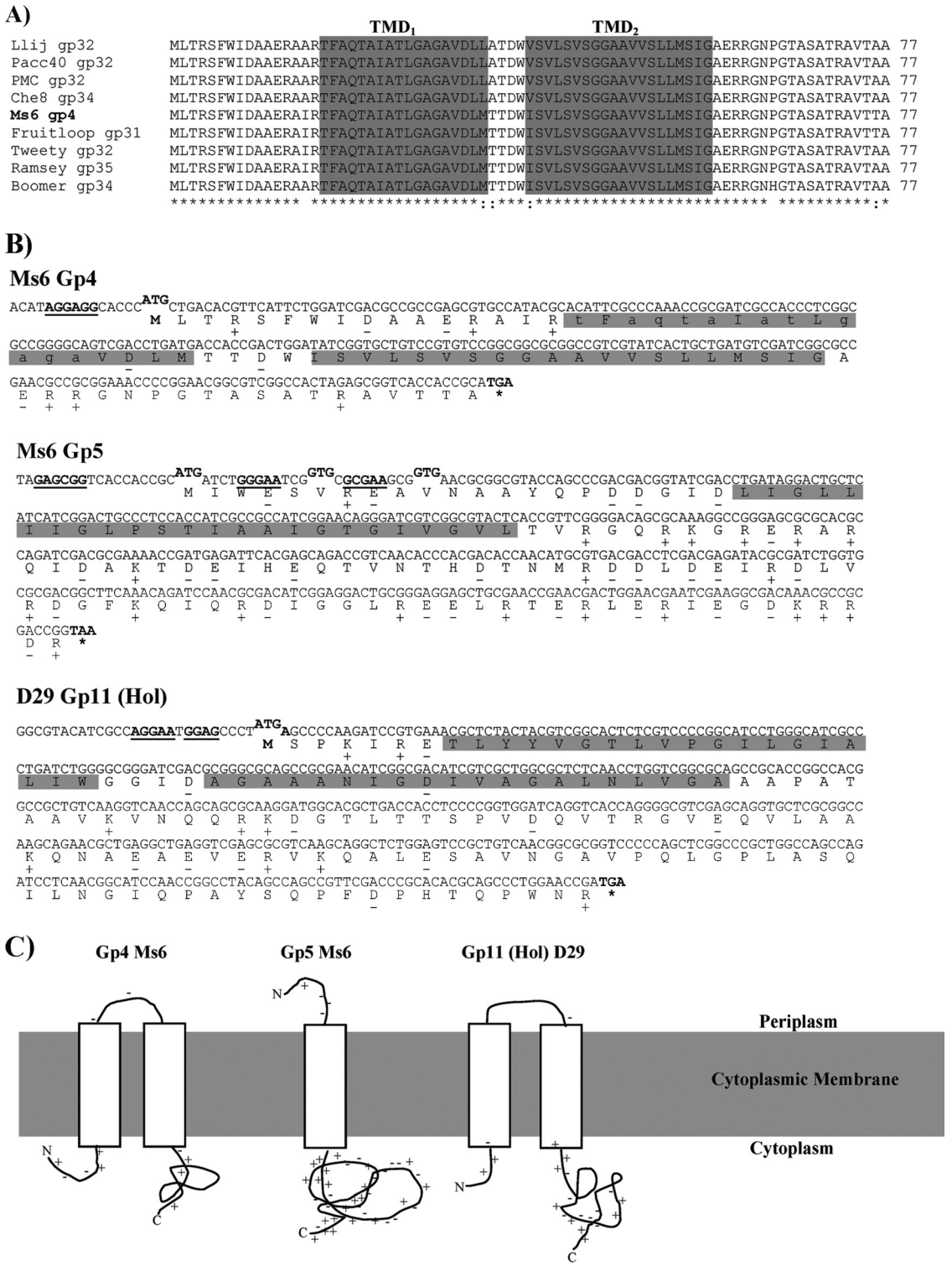

FIG. 1. Holin-like proteins of mycobacteriophages Ms6 and D29. (A) CLUSTALW alignment of Ms6 Gp4 (AAG48320) with similar sequences of Pham 95 members included in subcluster F1: Llij Gp32 (ABD58248), Pacc40 Gp32 (YP002241616), PMC Gp32 (ABE67533), Che8 Gp34 (NP817372), Fruitloop Gp31 (YP002241716), Tweety Gp32 (YP001469265), Ramsey Gp35 (YP002241822), and Boomer Gp34 (YP002014250) (the primary accession numbers of the UniProtKB/TrEmbl database are given in parentheses). Identical (*) and highly similar (:) amino acids are indicated. Numbers refer to the amino acid positions. The two TMDs are indicated in a gray box. (B) Sequences of genes coding for the class II holin (*gp4*) and class III holin (*gp5*) of Ms6 and class II holin of D29 (*gp11*). Charged residues are indicated by a plus or minus sign. TMDs are indicated in gray. Amino acid residues in the SAR domain of Gp4 that are predicted to be weakly hydrophobic are shown in lowercase. Potential translation start codons and corresponding Shine-Dalgarno sequences are in bold and underlined. (C) Topological model for Ms6 Gp4 (N-in, C-in), Gp5 (N-out, C-in), and D29 Gp11 (Hol) (N-in, C-in).

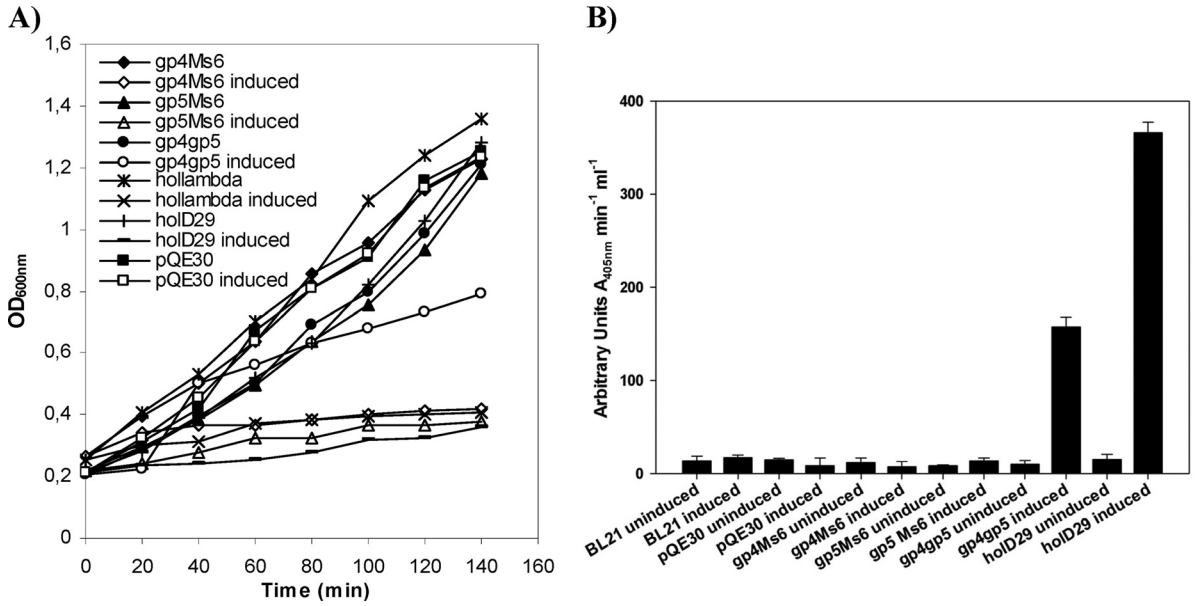

FIG. 2. (A) Expression of the holin-like proteins from mycobacteriophages Ms6 and D29 in *E. coli*. *E. coli* JM109 cells carrying plasmid pQE30 containing no insert or cloned genes were grown in LB broth at 37°C to an OD_{600} of 0.2. At time zero, transcription of cloned lysis genes was induced by addition of 1 mM IPTG. (B) Release of β-galactosidase from *E. coli* BL21 expressing Ms6 and D29 holin-like proteins. Activity was determined in the supernatants of induced cultures. Results are averages of triplicate experiments.

124-amino-acid polypeptide with a predicted molecular mass of 14.1 kDa. The *gp5* gene starts at an ATG codon that overlaps the *gp4* TGA stop codon in a different reading frame (4). A BLASTp search using the Ms6 Gp5 deduced amino acid sequence identified a number of putative proteins with unknown functions, restricted to the mycobacteriophage group of phages with a high degree of sequence identity. Owing to their related amino acid sequences, they have been recently grouped in mycobacteriophage gene phamily, Pham 96 (12). Analysis of the amino acid sequence of Gp5 using HMMTOP showed the presence of a single TMD membrane-spanning α-helix domain from residues 22 to 45 in the N terminus with a highly charged and hydrophilic C-terminal domain (Fig. 1B), structural characteristics of class III holins, and a predicted N-out, C-in topology (Fig. 1C). In addition to the location of Gp5 in the lysis cassette in the vicinity of the endolysin gene, overexpression of Gp5 in *E. coli* results in a drastic inhibition of cell growth (Fig. 2A), suggesting that the *gp5* gene product might function as a holin-like protein forming lesions in the cell membrane. However, in contrast to Ms6 Gp4, Gp5 was unable to complement a λS holin-defective mutant (data not shown).

Interestingly, there are three possible start codons in Ms6 *gp5* at positions 1, 6, and 10 that would produce 124 ($Gp5_{124}$), 118 ($Gp5_{118}$), and 114 ($Gp5_{114}$) amino acid products (of 14.1, 13.5, and 13 kDa, respectively), all preceded by potential ribosome-binding sites (5′-GAGCGG-3′ for $Gp5_{124}$, 5′-GGGA A-3′ for $Gp5_{118}$, and 5′-GCGAAG-3′ for $Gp5_{114}$). $Gp5_{118}$ and $Gp5_{124}$ have N-terminal extensions with one extra positively charged residue compared to $Gp5_{114}$, which could retard hole formation and also confer a negative-dominant antiholin character. However, unlike 21 S^{21} and Xfas53 *hol* whose translation is regulated by the presence of RNA stem-loop structures overlapping the ribosome-binding sites that reduce holin translation in favor of antiholin synthesis (23, 32), no RNA stem-loops were identified upstream of *gp5* mRNA.

Evaluation of holin lesion through β-galactosidase leakage. The ability of cytoplasmic membrane hole formation by Ms6 Gp4 or Gp5 was investigated by β-galactosidase leakage from *E. coli* BL21 strains expressing Ms6 holin-like proteins Gp4, Gp5, or both (Fig. 2A). This assay has been used to search for proteins with canonical holin-like activity, as the damage caused to the cytoplasmic membrane by the holin protein is sufficient to allow the leakage of cytoplasmic contents, including large proteins such as the constitutively expressed β-galactosidase enzyme (3). Our results show that a β-galactosidase leakage phenomenon (measured by an increase in enzymatic activity) was not observed when Gp4 or Gp5 expression was induced, suggesting that the lesions formed by these proteins are not large enough to allow the passage through the cytoplasmic membrane of proteins as large as β-galactosidase (Fig. 2B). In contrast to what is observed in Ms6 and other related mycobacteriophages, the lysis cassette of mycobacteriophage D29, a phage grouped in subcluster A2 (12), does not possess homologues of the accessory lysis protein Gp1 or Gp5. D29 lytic genes are clustered together, with the holin-like gene (*gp11*) localized between the *lysA* and *lysB* genes (12). The D29 *gp11* gene has the potential to code for a 141-amino-acid polypeptide with a predicted molecular mass of 14.6 kDa and possesses structural characteristics of class II holins, presenting two transmembrane domains from residues 8 to 26 (TMD1) and 31 to 50 (TMD2) (Fig. 1B), a highly charged and hydrophilic C-terminal domain, and N-in, C-in topology (Fig. 1C), as predicted by HMMTOP. In addition, D29 Gp11 (Hol) overexpression in *E. coli* inhibits cell growth as observed for holin-like proteins (Fig. 2A). As shown in Fig. 2A, induction of D29 Hol expression allowed the release of β-galactosidase to the

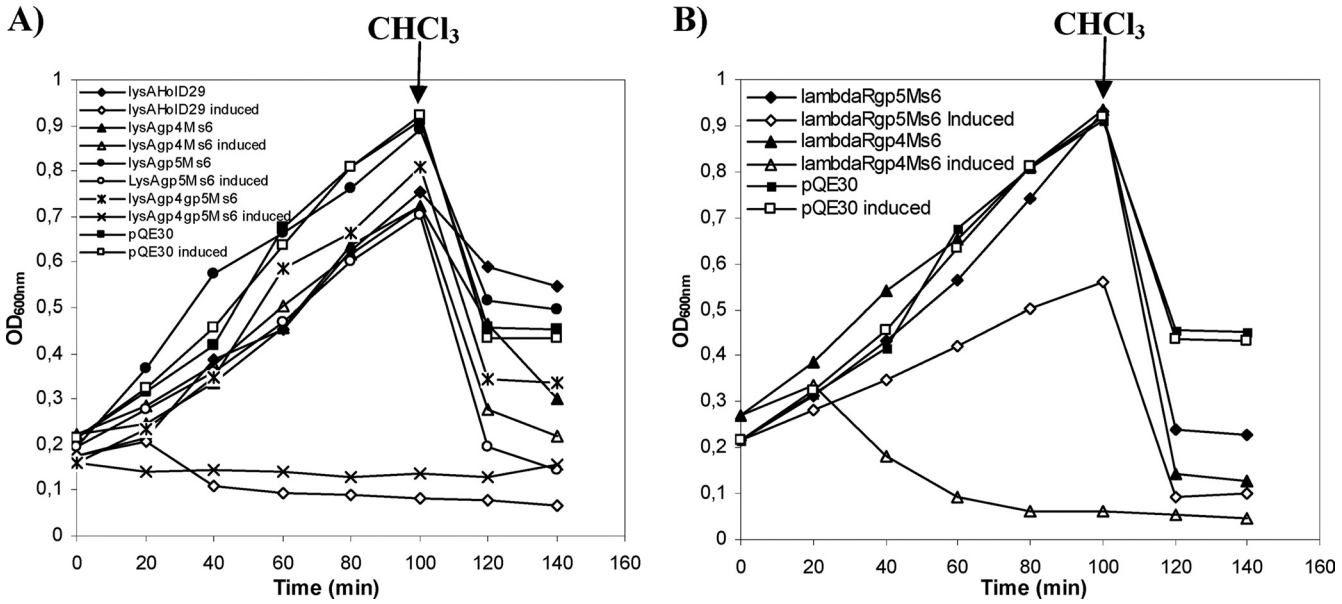

FIG. 3. (A) Effect of the expression of phage endolysin/holin pairwise combinations on *E. coli* growth. (B) Effect of the expression of pairwise combinations of the λR endolysin with the Ms6 holin-like proteins Gp4 and Gp5 in *E. coli*. *E. coli* JM109 cells carrying plasmid pQE30 containing no insert or cloned genes were grown in LB broth at 37°C to an OD_{600} of 0.2. At time zero, transcription of cloned lysis genes was induced by addition of 1 mM IPTG. At the time indicated by the arrow, 2% $CHCl_3$ was added to the cultures.

culture medium, resulting in an increase of the enzymatic activity (377 arbitrary OD_{405} units ml^{-1} min^{-1}) by comparison to the BL21 control cells (14 arbitrary OD_{405} units ml^{-1} min^{-1}) (Fig. 2B). These results suggest that the D29 holin (Gp11) functions as a canonical holin, forming large lesions in the cytoplasmic membrane sufficient to allow the access of the cytoplasmic endolysin to the peptidoglycan and bring about effective lysis of the host. These data support a potential holin function for D29 Gp11 (Hol) as previously proposed (12, 25). Surprisingly, the concomitant expression of Ms6 Gp4 and Gp5 results in a moderate increase in the β-galactosidase activity (156 arbitrary OD_{405} units ml^{-1} min^{-1}), suggesting that these two proteins together may form a larger hole lesion than the sole proteins.

Expression of holin/endolysin pairwise combinations in *E. coli*. In contrast to what happens with phage endolysins that possess a narrow range of activity regarding the infected bacteria, holins are not species specific and do not specifically interact with the endolysins (29, 41). Since canonical holins, such as λS, form very large nonspecific holes that allow fully folded unrelated endolysins to pass through the membrane and attack the murein, we expressed in *E. coli* different pairwise combinations of endolysins/holins in an attempt to clarify the role of Ms6 Gp4 and Gp5 in bacterial lysis. We observed that the concomitant expression of Ms6 LysA with Gp4 or Gp5 was not sufficient to support a lysis phenotype in *E. coli*. However, coexpression of LysA with both Gp4 and Gp5 resulted in a slight decrease of the OD_{600} 40 min after induction (Fig. 3A). The fact that the lack of a lysis phenotype was a consequence of the inability of Ms6 Gp4 or Gp5 to form lesions on the cytoplasmic membrane large enough to allow the passage of LysA to the periplasm was further confirmed by concomitantly expressing the Ms6 endolysin LysA with the D29 Gp11 holin. Lysis of *E. coli* was observed beginning 20 min after induction, which suggests that the D29 holin is functional in *E. coli* and allows the access of Ms6 LysA to the peptidoglycan (Fig. 3A). In addition, the Ms6 Gp4 but not Gp5 allows the access of the λ transglycosylase to the murein, as demonstrated by complementation of a λS mutant (4; M. J. Catalão, unpublished data), and unlike Gp4, Gp5 was unable to promote release of λR, the cytosolic endolysin of phage λ (Fig. 3B). We interpret this to mean that the Ms6 Gp4 or Gp5 alone makes holes too small to allow the passage of Ms6 LysA in *E. coli*.

Cross-linking of Gp4 and Gp5 in the *E. coli* cell membrane. It is known that holins must oligomerize to achieve their lethal membrane effect (9). To identify the oligomeric states of Ms6 Gp4 and Gp5, the membrane fractions from *E. coli* expressing Gp4 or Gp5 (fused to an S tag at the N terminus and a His_6 tag at the C terminus) from a derivative plasmid of pET29a were collected 60 min after induction; proteins were extracted from the membranes with Triton X-100 and subjected to cross-linking with the water-soluble membrane-impermeant, homobifunctional sulfo-*N*-hydroxy-succinimide ester, BS^3. The presence of a band of 24.2 kDa in the absence of the cross-linker shows that Gp4 forms SDS-resistant dimers during membrane extraction with Triton X-100 (Fig. 4A). Furthermore, Gp4-specific bands corresponding to molecular masses of 12.1, 24.2, 36.3, 48.4, and 60.5 kDa, up to pentamers, could be detected by Western blot analysis (Fig. 4A). In contrast, Gp5, which does not complement a λS-defective mutant phage, formed only trimers but not higher oligomers under the same conditions (Fig. 4B). This result might help explain the inability of Gp5 to support an efficient lysis of *E. coli* when it is coexpressed with different endolysins as the ability of holin molecules to oligomerize is essential for the lytic step in holin function (8, 9, 43).

Evidence for an interaction between Gp4 and Gp5. Despite the toxicity observed after expression induction of Gp4 or Gp5

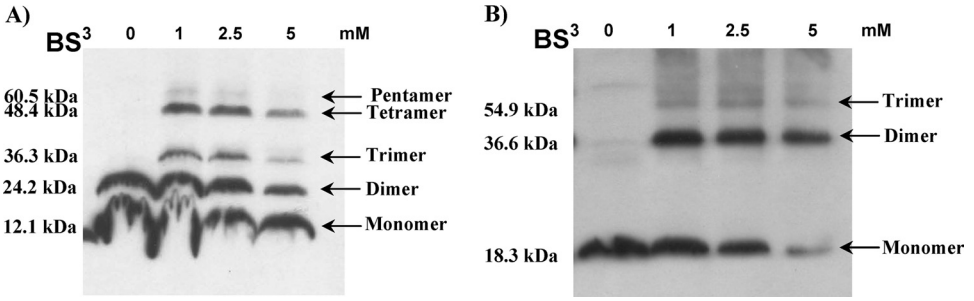

FIG. 4. Ms6 Gp4 (A) or Gp5 (B) oligomerization. Proteins from membranes of *E. coli* BL21(DE3) expressing Gp4 or Gp5 were extracted with Triton X-100 and treated with different BS^3 concentrations as described in Materials and Methods. Proteins were detected by Western blotting with an anti-His_6 antibody. Predicted molecular masses are indicated to the left of the panels. Oligomerization bands are indicated by arrows.

membrane proteins in *E. coli*, simultaneous expression of these proteins attenuates the lethal effect (Fig. 1A). For bacteriophage lambda, it has been proposed that the lysis inhibitor S_{107} (a protein of 107 amino acids encoded by the *S* gene) inhibits lysis through intermolecular interaction with the lysis effector S105 (10). Accordingly, the ability of Gp5 to inhibit the Gp4 lethal effect suggests that it may interact with the holin. To demonstrate this, both proteins were expressed from the same vector in *E. coli* with Gp4 fused to an S tag at the N terminus and Gp5 fused to a His_6 tag at the C terminus. At 60 min postinduction, cell membranes were collected by centrifugation, proteins were extracted with 1% Triton X-100, and this fraction was subjected to chemical cross-linking with BS^3. In the absence of the cross-linker, we detected a band with a molecular mass of 14.9 kDa, corresponding to the size predicted for Gp5-His_6 monomer, and a faint band of ~26 kDa corresponding to an interaction between an S-tagged Gp4 monomer and a Gp5-His_6 monomer (Fig. 5). Cross-linking using 1 mM BS^3 revealed an additional band of 30 kDa corresponding to the Gp5 dimer. The increase in BS^3 concentration to 5 mM led to the appearance of a band of ~53 kDa as a result of oligomer formation between the Gp5 homodimer and Gp4 homodimer. This result suggests that Gp5 interacts with the Ms6 holin in some cooperative fashion to effect lysis and suggests that it may control Gp4 function during the lytic cycle.

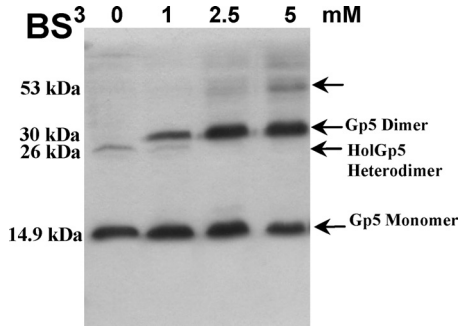

FIG. 5. Interaction between Gp4 and Gp5 of mycobacteriophage Ms6. Cross-linking and sample preparation for Western blot analysis was performed as described in the legend of Fig. 4. Proteins were detected with an anti-His_6 antibody. In the absence of BS^3, Gp5 monomer and Gp4-Gp5 heterodimer are detected. Predicted molecular masses are indicated to the left of the panels. Oligomerization bands are indicated by arrows.

Construction of Ms6 mutants defective for lysis. Concerning the possibility that Ms6 Gp4 and Gp5 may behave differently in the heterologous *E. coli* host and in the Ms6 natural host, *M. smegmatis*, we constructed Ms6 mutants defective for synthesis of Gp4, Gp5, or both proteins. Using the bacteriophage recombineering of electroporated DNA (BRED) system (19), we constructed internal in-frame deletions of Ms6 *gp4*, *gp5*, or *gp4* and *gp5* in the Ms6 wild-type phage and, in a second step, of the *gp1* gene in the previously constructed mutants defective for Gp4 or Gp5 synthesis ($Ms6_{\Delta gp4}$ or $Ms6_{\Delta gp5}$) by allelic gene replacement. Even though we have not yet been able to recover a purified mutant derivative of phage $Ms6_{\Delta gp4\ gp5}$, probably reflecting poor viability of the mutant, pure mutants of $Ms6_{\Delta gp4}$ and $Ms6_{\Delta gp5}$ were readily identified in high frequencies and in the absence of any selection (Fig. 6). In view of the fact that the absence of Gp4 or Gp5 from the Ms6 virion has no apparent effect on phage viability and based on our recent observations that the accessory lysis protein Gp1 is required for a normal burst of infective phage particles (2), we further investigated its function during the Ms6 lytic cycle. We constructed an internal in-frame deletion of the *gp1* gene in defective phage $Ms6_{\Delta gp4}$ and $Ms6_{\Delta gp5}$ using the same recombineering strategy described above, and pure mutants of $Ms6_{\Delta gp1\ gp4}$ and $Ms6_{\Delta gp1\ gp5}$ were isolated after PCR screening of secondary individual plaques (Fig. 6).

These results demonstrate that $Ms6_{\Delta gp4}$, $Ms6_{\Delta gp5}$, $Ms6_{\Delta gp1\ gp4}$, and $Ms6_{\Delta gp1\ gp5}$ are viable, that neither *gp4* nor *gp5* is essential for plaque formation, and that an Ms6 mutant phage lacking the *gp4* and *gp5* lysis genes is able to infect and lyse mycobacteria even though it could not be isolated yet.

Ms6 holin-like proteins are not required for *M. smegmatis* lysis. One-step growth curves and determination of phage growth parameters (latent period, rise period, and burst size) were carried out to compare the Ms6 mutant replication cycle. Results are summarized in Table 2. The one-step growth experiment (Fig. 7A) shows that Gp4 and Gp5, although nonessential for lysis, have an effect on the lysis timing since an Ms6 *gp4* deletion mutant caused slightly accelerated lysis (80 min), whereas an Ms6 *gp5* deletion mutant delayed lysis (170 min), which is consistent with holin function. These lysis times correspond to the latent time represented in Fig. 7A in addition to the initial 50 min of adsorption and were compared to the Ms6

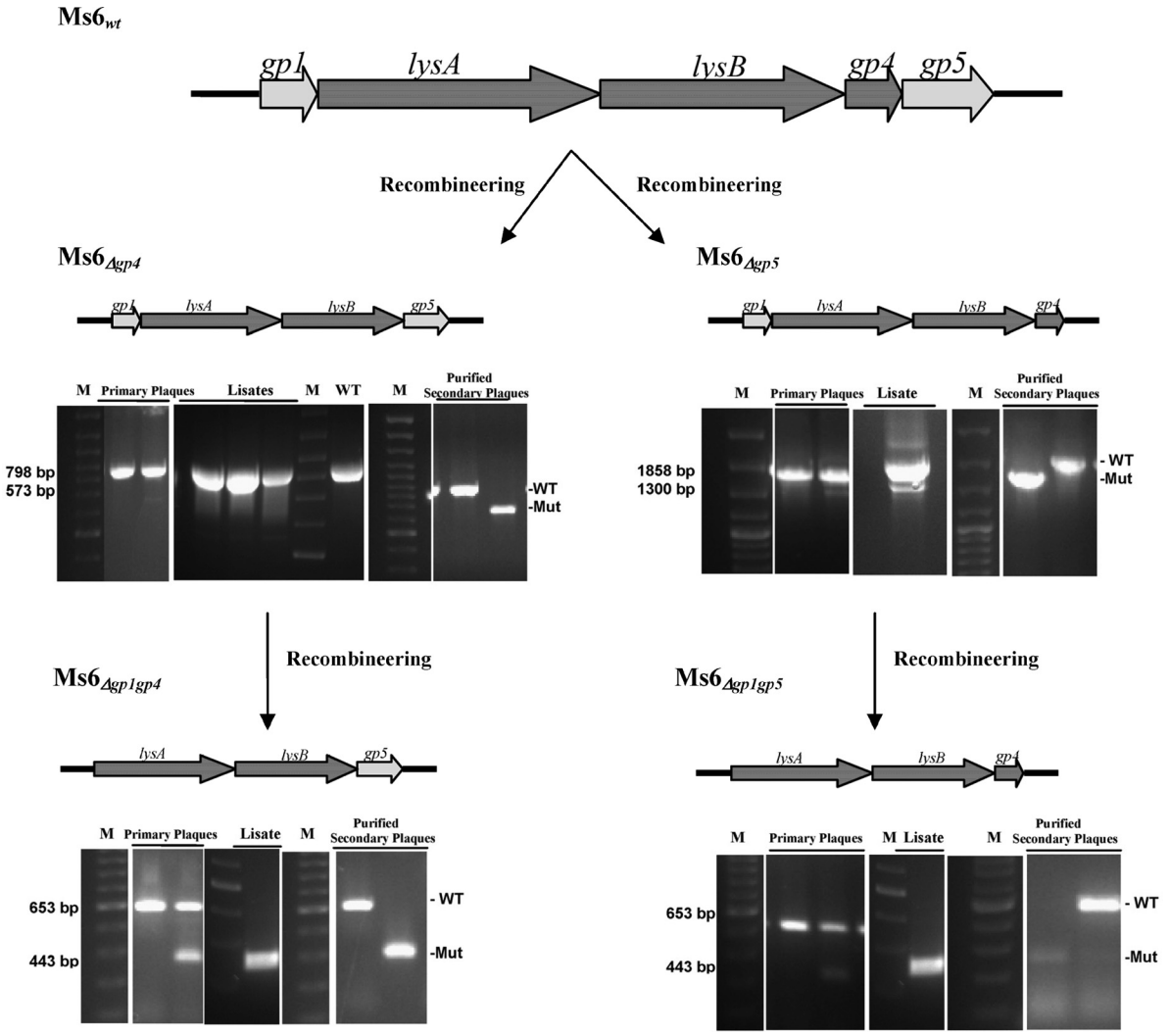

FIG. 6. Strategy for construction of Ms6 lysis gene deletion mutants. A 200-bp dsDNA substrate that has 100-bp homology flanking the deletion was designed. Following coelectroporation of the 200-bp substrates and genomic DNA (of $Ms6_{wt}$ to obtain $Ms6_{\Delta gp4}$ and $Ms6_{\Delta gp5}$ mutants or of $Ms6_{\Delta gp4}$ and $Ms6_{\Delta gp5}$ to obtain $Ms6_{\Delta gp1\ gp4}$ and $Ms6_{\Delta gp1\ gp5}$ mutants), primary plaques were recovered to identify a mixed plaque containing wild-type and mutant phages. 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 homogenous deletion mutants.

wild-type phage (110 min) under the same experimental conditions. Thus, the absence of *gp4* or *gp5* in the infecting virion has an evident effect on the timing of lysis. Single-burst experiments were done to compare the viable progeny released from single cells infected with $Ms6_{wt}$ or the phage mutants. The number of infective particles released after infection with the $Ms6_{\Delta gp4}$ phage is lower than in an $Ms6_{wt}$ infection, whereas after infection with the $Ms6_{\Delta gp5}$ phage, an increase in the burst size was detected. Under our experimental conditions, when $Ms6_{wt}$ infects *M. smegmatis* mc^2155, there is an average of 149 viable phage released from one bacterium while infection with $Ms6_{\Delta gp4}$ or $Ms6_{\Delta gp5}$ yielded an altered burst size of approximately 115 or 221 viable phage per infected cell, respectively. Deletion of the *gp1* gene from $Ms6_{\Delta gp4}$ or $Ms6_{\Delta gp5}$ drastically reduced the burst size to ~28 phage/infected cell or ~77 phage/infected cell for $Ms6_{\Delta gp1\ gp4}$ or $Ms6_{\Delta gp1\ gp5}$ phage, respectively, which is in agreement with our previous results, which showed that Gp1 is essential to achieve the wild-type burst (2). When we analyzed the plating ability and the plaque size of the lysis-defective phage, we observed that all except $Ms6_{\Delta gp5}$ produced smaller plaques, with no size variation, than

TABLE 2. Viability and phage growth parameters of mycobacteriophage Ms6 and lysis gene deletion derivatives

Phage	Plaque-forming ability	Burst size (no. of phage)	Latent time (min)	Plaque size (mm)
$Ms6_{wt}$	Yes	149 ± 32	110	2–3
$Ms6_{\Delta gp1}$	Yes	45 ± 13	110	~1
$Ms6_{\Delta gp4}$	Yes	115 ± 39	80	1–2
$Ms6_{\Delta gp5}$	Yes	221 ± 56	170	4–5
$Ms6_{\Delta gp1\ gp4}$	Yes	28 ± 6	140	1–2
$Ms6_{\Delta gp1\ gp5}$	Yes	77 ± 27	200	<1
$Ms6_{\Delta gp4\ gp5}$	Yes			
$Ms6_{\Delta gp1\ gp4\ gp5}$	No			

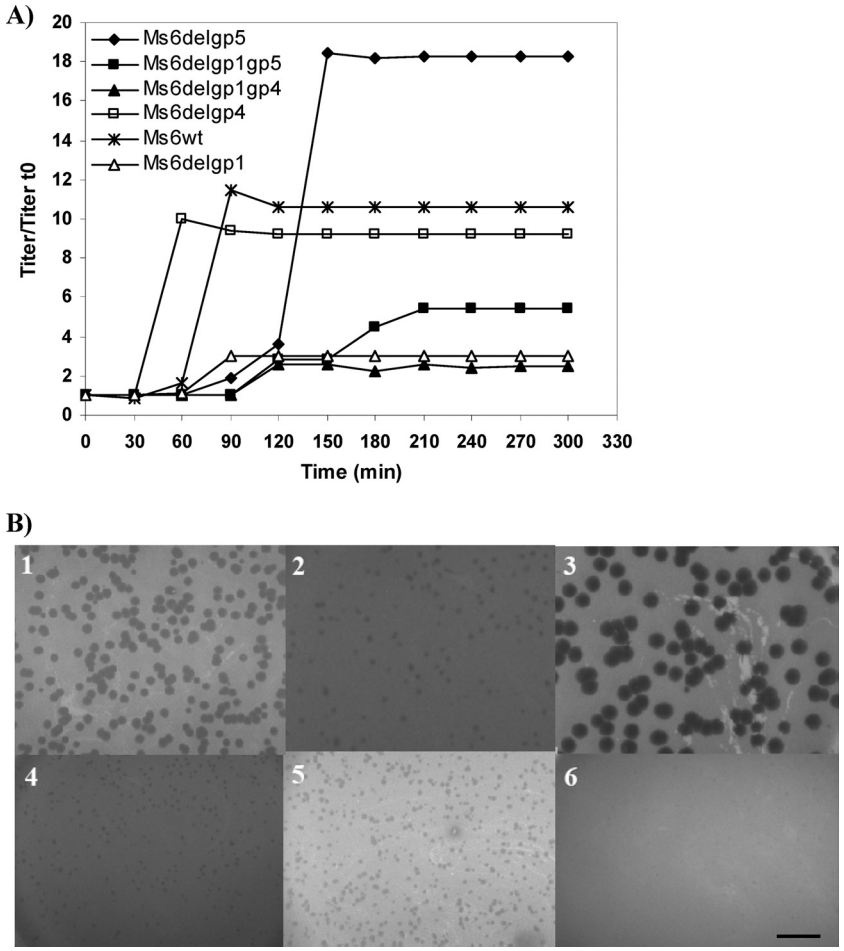

FIG. 7. (A) One-step growth curves of mycobacteriophage Ms6 and lysis gene deletion derivatives. For each curve the titers measured were divided by the titer at time zero (t_0) for normalization (titer/titer t_0). Results are averages of three independent experiments. (B) Plating ability of the different lysis gene mutant bacteriophage. Images show results of *M. smegmatis* infection with the following: frame 1, $Ms6_{wt}$ phage; frame 2, $Ms6_{\Delta gp4}$; frame 3, $Ms6_{\Delta gp5}$; frame 4, $Ms6_{\Delta gp1}$; frame 5, $Ms6_{\Delta gp1\ gp5}$; frame 6, $Ms6_{\Delta gp1\ gp4}$. Scale bar, 1 cm.

those produced by $Ms6_{wt}$ phage (Fig. 7B). The size of the plaques produced by $Ms6_{\Delta gp1\ gp4}$ was <1 mm, whereas the plaques produced by the $Ms6_{\Delta gp5}$ mutant were very large, with diameters of 4 to 5 mm, in agreement with a lower and a larger burst size, respectively, than $Ms6_{wt}$ phage. Taken together, these results suggest that in addition to *gp4*, *gp5* encodes a holin-like protein, and they must act in concert to control the timing of lysis. Furthermore, as previously observed (2), the presence of Gp1 is crucial to obtain a normal burst of infective phage although it has no influence on duration of the latent time of the lytic cycle.

DISCUSSION

Even though the mechanisms underlying mycobacteriophage lysis of mycobacteria are poorly understood, recent work has contributed significantly to the progress in the field (2, 5, 6, 14, 25). Nonetheless, the exact mechanism by which the lysis effectors LysA and LysB are localized to their substrates remains elusive in the majority of the mycobacteriophages. Very recently, we have identified the product of Ms6 *gp1* gene as a chaperone-like protein that specifically interacts with the endolysin and is involved in its translocation across the cytoplasmic membrane (2). Moreover, removal of Gp1 function in mycobacteriophage Ms6 showed that, although not essential for plaque formation, the protein is required for efficient phage release. Similarly to what has been reported for phages possessing endolysins endowed with signal sequences or SAR domains, Ms6 LysA translocation in *E. coli* also involves the host Sec system (2). These data, together with the previous reported absence of lysis when Ms6 LysA and Gp4 were coexpressed in *E. coli* (4), led us to investigate the function of the previously identified holin protein in the mycobacteriophage Ms6 infection context. The *gp4* gene is localized downstream of *lysB* and encodes a protein with structural characteristics of class II holins with the ability to complement a λS defect. In phages like the lambdoid 21, where the endolysin possesses a SAR domain and translocation is holin independent, holins belong to a recently discovered class of proteins, the pinholins, that make small holes in the host membrane that are sufficient to depolarize it and allow membrane release of SAR endolysins even though they are not large enough to allow escape of canonical cytoplasmic endolysins. In these cases

the holin function is restricted to regulation of the timing of lysis (21, 23).

Reexamination of the predicted amino acid sequence of Ms6 Gp4 showed that its TMD1 has characteristics of a SAR domain with a high percentage of hydrophobic residues, a characteristic described for the pinholin of the lambdoid phage 21 (22). Although Gp4 was unable to support the Ms6 LysA-mediated lysis of *E. coli* cells (4), unlike the pinholin of phage 21 S^{21} (23), it was able to promote the release of R, the cytosolic endolysin from phage λ (Fig. 2B), but not the release of Ms6 LysA or D29 LysA. Of note is the fact that the predicted molecular mass of the Ms6 endolysin is 43 kDa while λR is a protein of 17.8 kDa, which suggests that the passage of endolysins through holin holes is dependent on membrane pore size.

Gene organization in bacteriophage lysis cassettes may be extremely diverse: for the majority of phages, lysis genes are clustered and transcribed in the order of holin first and then endolysin, as exemplified by lambda phage (39). An inverted organization (*lys* upstream of *hol*) was reported for the *Oenococcus oeni* bacteriophage fOg44 (24), and in many cases the genes are not even linked (e.g., T4) (29). In many phages of *Streptococcus thermophilus* (30) and in phage Av-1, which infects the Gram-positive bacteria *Actinomyces naeslundii* (3), two putative holin genes precede the endolysin gene. In *Bacillus subtilis* prophage PBSX, it was proposed that two open reading frames preceding the endolysin *xlyA*, designated *xhlA* and *xhlB*, encode polypeptides that associate in the membrane to form a functional holin complex that allows XlyA access to the peptidoglycan (15, 18). In *Staphylococcus aureus* phage P68, a putative holin gene, *hol15*, was identified in the −1 reading frame at the 3′ and of the endolysin gene *lys16*. A second putative holin gene, *hol12*, was later identified at the end of the structural genes (33). In some phages, like λ and 21, the holin gene presents a dual-start motif producing two proteins by virtue of alternate translational starts, the holin and the antiholin, while in other phages these two proteins are encoded by separate genes (e.g., P1 and T4) (29, 42). Such diversity is also observed in mycobacteriophages: in addition to the endolysin LysA, the majority of mycobacteriophages sequenced so far encodes an additional enzyme with lipolytic activity, LysB, that targets the outer membrane of mycobacteria (5, 6). In phages belonging to cluster A2, like D29, the holin gene is positioned between *lysA* and l*ysB* while in phages belonging to cluster F1, as in the Ms6 case, the holin gene is localized immediately downstream of *lysB*. For many mycobacteriophages a holin gene has not been identified yet, while in others (Ms6 and other members of subcluster F1) an additional lysis gene (*gp5*) encoding a predicted membrane protein is positioned immediately downstream of the *gp4* gene. Gp5 encodes a 124-amino-acid protein possessing a single TMD and a very highly charged and hydrophilic C-terminal domain, and we hypothesized that it might function as a holin-like protein. Gp5 was found to be unable to support both LysA- or λR-mediated lysis in *E. coli*, and, in contrast to Gp4, oligomerization appeared to be blocked at the trimer stage in detergent (as for Gp4, the final degree of oligomerization is not yet known). Despite the toxicity observed when the integral membrane proteins Gp4 and Gp5 are independently expressed in *E. coli*, simultaneous expression of these proteins attenuates the lethal effect, which suggested that they may interact. For bacteriophage lambda, it has been proposed that the lysis inhibitor S107 inhibits lysis through dimeric interactions with the lysis effector S105 (10). Indeed, using chemical cross-linking, we were able to obtain biochemical evidence for a direct interaction between Gp4 and Gp5; however, the exact mechanism by which Gp5 acts to control Gp4 function remains elusive. Owing to the concerns that exist when holin genes are expressed from strong inducible promoters, we constructed different Ms6 mutants with deletions of the holin-like genes. Indeed, it is well known that a membrane protein overproduced from a multicopy plasmid can insult the membrane sufficiently to cause release of cytoplasmic endolysins (42). Although more time-consuming, this strategy has two important advantages: (i) it allows the function of these proteins to be examined in their natural host, and (ii) each gene product in the cell, resulting from phage infection, is produced at physiological levels. When we analyzed the holin gene-deleted mutant infection cycle by one-step growth curves, we observed that the gene products of *gp4* and *gp5*, although nonessential for phage viability, appear to play a role in controlling the timing of lysis. $Ms6_{\Delta gp4}$ caused accelerated lysis, whereas $Ms6_{\Delta gp5}$ delayed lysis, which is consistent with holin function. We also considered whether Gp5 could act as an antiholin. Antiholins generally delay phage lysis in order to optimize progeny phage production. This should have been observed if Gp5 acts as an antiholin, and its absence would result in earlier lysis and smaller plaques due to premature lysis. Unexpectedly, this phenotype was observed when Gp4 was deleted from the lytic cassette. In contrast, deletion of Gp5 delayed lysis and resulted in very large plaques due to an increase in the burst size. Not all dsDNA phages utilize an antiholin to regulate lysis timing since some apparently simply rely on delaying expression of their holin genes (16). These results suggest that mycobacteriophage Ms6 *gp4* and *gp5* encode holin proteins whose combined action could play the role of a holin and that expression of both proteins is necessary to effect host cell lysis at the correct and programmed timing, as described for other phages such as the *A. naeslundii* phage Av-1 (3) and the *B. subtilis* PBSX phage (15). Moreover, interaction of Gp5 with Gp4 may contribute to very precise adjustment of the timing of hole formation and to keeping the infected cell productive, allowing the assembly of more virions. The ubiquity of holin-mediated lysis systems results from the ability of phages to rapidly evolve to shorter or longer infection cycles to adjust to changes in host quality or density (37). Deletion of the chaperone-like protein *gp1* in both deletion mutants $Ms6_{\Delta gp4}$ and $Ms6_{\Delta gp5}$ was catastrophic for lysis, with more than 3-fold reduction of the burst size, even though the mutant phage are viable and could be isolated. Remarkably, although Gp5 was unable to allow endolysin-mediated lysis in *E. coli*, a mutant phage lacking both *gp1* and *gp4* was able to infect *M. smegmatis* cells and undergo lysis, so it is expected that there will be alternative pathways to release phage progeny (13). We conclude that for mycobacteriophage Ms6 and related mycobacteriophages, the presence of the endolysin in addition to one of the lytic genes, *gp1*, *gp4*, or *gp5*, is sufficient for a lysis phenotype. However, this results in dramatic changes in the infective cycle and in lower viability of the mutant phage. The presence of the mycobacterium-specific lysis factors Gp1 and Gp5 that are restricted to mycobacteriophages (4, 12) may

confer a selective advantage not only for fitness under different environmental conditions but also as an alternative to exclusively holin-dependent lysis; it has been shown that single missense changes within the holin proteins can have a profound effect on both the process of host lysis and its timing, unpredictably resulting in dramatic shortening or lengthening of the infection cycle (8, 42). In addition, holin membrane holes have different sizes, and for holes too small to allow the passage of endolysins, phages must evolve in order to survive. Some phages evolved by synthesizing secreted endolysins endowed with signal sequences, while mycobacteriophages throughout their evolution acquired additional lysis genes which may confer host lysis benefits and successful phage propagation and replication. With this study we hope to have contributed to a better understanding of lysis timing regulation by mycobacteriophages.

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