

GP30 of the mycobacteriophage CASbig impairs mycobacterial adaptation during acidic stress and in macrophages

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Abstract: The rapid emergence of multidrug-resistant and extensively drug-resistant Tuberculosis retrieved intense interest in phage-based therapy. This old approach, which was abandoned in the west in the 1940s but is generating renewed interest, has stimulated fresh research on mycobacteriophages and their lytic efficiency against their hosts. GP30 is a novel protein of the mycobacteriophage CASbig with undiscovered function. In this study, we analyzed the role of CASbig gp30 in the host *Mycobacterium smegmatis*. Overexpression of gp30 in the host led to reduced growth in acidic medium and attenuated the intracellular survival rate of *M. smegmatis* inside the THP-1 macrophages, which may be linked to the altered lipid profile of the recombinant bacterial cell wall. In a word, this study suggested that gp30, a novel gene from a mycobacteriophage, modulated lipid composition and content to hamper the survivability of bacteria under stress conditions.

Introduction

Accounting for 1.5 million deaths from tuberculosis (TB) in 2018, *Mycobacterium tuberculosis* (Mtb) remains a frightful threat to public health worldwide (World Health Organization, 2018). The epidemic of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB aggravated this situation (Coll *et al.*, 2018; Velayati *et al.*, 2018). To combat this predicament, the identification of new therapeutic or synergistic targets that are able to augment the antimicrobial efficiency against TB is an urgent need.

The demand for new methods to overcome drug-resistant Mtb has stimulated fresh research on mycobacteriophages and their lytic efficiency against their hosts (Oldfield and Hatfull, 2014; Sassi *et al.*, 2014). There are many small phage gene-encoded proteins or peptides with direct and/or indirect host bacteria-killing/inhibiting functions. Good examples are found in different bacteria such as *Bacillus anthracis* (Schuch *et al.*, 2002; Yoong *et al.*, 2006), *Streptococcus pneumoniae* (Loeffler *et al.*, 2001), *Staphylococcus aureus* (Liu *et al.*, 2004), *Mycobacterium ulcerans* (Fraga *et al.*, 2019), etc. Recently, using

bacteriophage genomics and peptidomics approaches, Wei *et al.* (2013) identified a mycobacteriophage peptide, PK34 that had direct Mtb-killing activity. Preliminary comparative analysis of GP30, a protein identified in a novel virulent mycobacteriophage, CASbig (Teng *et al.*, 2015), showed its maximum identity (36.4%) to PK34 peptide, indicating the probable role of GP30 in host bacteria-killing function.

To demonstrate this hypothesis, the function of the gp30 gene was studied by the construction of recombinant *Mycobacterium smegmatis* expressing GP30 (MS_gp30) and *M. smegmatis* carrying empty vector only (MS_Vec) as a control strain. The results indicated that GP30 impairs the survival rate of *M. smegmatis* under acidic stress. We also detected that GP30 was able to reduce mycobacterial intracellular survival, alter the surface architecture and fatty acids profile of recombinant *M. smegmatis*.

Materials and Methods

Bacterial strains and growth conditions

The human leukemic monocyte lymphoma cell line (THP-1) was purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. *M. smegmatis* mc² 155 strain was a kind gift from the Institute of Modern Biopharmaceuticals, Southwest University, Chongqing, China. *M. smegmatis*

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mc²155 and its derivative strains were cultured on Middlebrook 7H10 agar medium (BD Difco, USA) supplemented with 0.5% glycerol. For liquid cultures, *M. smegmatis* strains were cultured in Middlebrook 7H9 broth added 0.2% (w/v) glucose, 0.05% (v/v) Tween-80 and 0.2% (v/v) glycerol or Sauton's media added 0.05% (v/v) Tween-80. *Escherichia coli* strains were cultured in Luria-Bertani (LB) fluid medium. If needed, kanamycin was added at the following concentrations: 20 µg/mL for mycobacterial strains and 50 µg/mL for *E. coli*. All bacteria were incubated at 37°C.

Over-expression of a Myc-GP30 fusion protein in *M. smegmatis*

The gp30 gene was PCR amplified from the mycobacteriophage CASbig genome using the specific forward primer 5'CACGAGAATTCATGAAACCGAC TGCACGC3' with an *EcoRI* site (bold) and the specific reverse primer 5'CTATAGGATCCCTTGCCCCACCTC CGA3' with a *BamHI* site (bold). The PCR product of approximately 150 bp was inserted into the expression plasmid pNIT-myc (Li et al., 2014; Pandey et al., 2009) to construct the recombinant pNIT-gp30 plasmid. The recombinant vector was confirmed by restriction enzyme digestion as well as automated DNA sequencing (BGI, China). The recombinant vector pNIT-gp30 was electroporated in electro-competent *M. smegmatis* mc² 155 cells to generate recombinant *M. smegmatis* (MS_gp30), and the transformants were selected on 7H10 agar medium with 20 µg/mL kanamycin. *M. smegmatis* mc² 155 harbored the empty pNIT-myc vector was used as a control group (named as MS_Vec). MS_Vec and MS_gp30 were induced with 28 mM ε-caprolactam, harvested by centrifugation and lysed by ultrasonication. Protein samples were separated by SDS-PAGE, and the expression of Myc-GP30 fusion protein was confirmed by immunoblotting using a mouse anti-Myc antibody (Beyotime Biotechnology, China).

Bacterial growth curves and acidic challenge

For *in vitro* growth kinetics, MS_gp30 and MS_Vec were cultured in 100 mL of 7H9 media with a starting absorbance of ~0.02 at 600 nm. 28 mM ε-caprolactam as an inducer was added to the broth, the OD600 was monitored at regular intervals of 6 or 12 h, and the mean value was applied to create growth curves. To measure the growth kinetics of MS_gp30 and MS_Vec under acidic stress, growth velocity was measured by determining the OD600 at regular intervals of 6 or 12 h. Experiments were performed in triplicate, and the mean value was applied to create growth curves.

Spot tests

MS_gp30 and MS_Vec, induced by 28 mM ε-caprolactam, were treated with acidic conditions for 6 h, then tenfold serially diluted and spotted onto 7H10 agar. A Nikon COOLPIX P300 digital camera was used to take photographs of bacterial lawns after 3–4 days of cultivation at 37°C. The experiments were repeated at least three times.

In vitro survival curves

The recombinant strains MS_gp30 and MS_Vec were cultivated in 7H9 broth until the OD600 reached around 1.0. Then, the recombinant bacteria were reinoculated in normal or acidic 7H9 media, and the OD600 of the cultures

was adjusted to 0.5. After the indicated time, cell survival was monitored by a colony formation assay on 7H10 agar media supplemented with kanamycin. Colony-forming units (CFU) were counted every 12 or 24 h after treatment with several stresses. Three independent experiments were conducted for each acidic condition.

Mycobacterial lipid extraction and fatty acid analysis

The mid-exponential phase cultures of MS_Vec and MS_gp30 fatty acid were extracted as previously described (Lewis et al., 2000). The fatty acid concentrations were measured by gas chromatography-mass spectrometry (GC-MS) (Thermo Fisher Trace GC 1310-ISQ LT single quadrupole EI MS, A1-1310 autosampler) with a Thermo TG-5MS capillary column. The detailed analytical procedure has been described previously (Luo et al., 2016).

Scanning electron microscopy (SEM) analysis

The recombinant strains, MS_gp30 and MS_Vec, were cultivated for approximately 24 h until the OD600 reached 0.9 in 7H9 broth containing 28 mM ε-caprolactam. Bacteria were harvested and resuspended in 2.5% glutaraldehyde solution. The recombinant strains, MS_gp30 and MS_Vec, were dehydrated in an ascending series of ethyl alcohol. All samples were sputter-coated with platinum (IB-3, Eiko) after critical point drying and observed by SEM (JSM-6390 LV, JEOL Ltd.).

Intracellular survival assay

Intracellular survival assay was performed as previously described (Li et al., 2016; Li et al., 2014). THP-1 cells were cultured in RPMI-1640 (Hyclone) medium containing 2 mM L-glutamine, supplemented with 10% fetal calf serum (Hyclone), 100 U/mL penicillin, and 100 U/mL streptomycin. Suspension cultures of THP-1 cells were seeded at 1×10^6 cells per well in 12-well tissue culture plates (Corning) and transformed into an adherent state in the presence of 100 ng/mL Phorbol-12-myristate-13-acetate (PMA; Sigma) at 37°C in 5% CO₂ for 48 h. 250 nM isovaleronitrile (Sigma) in DMSO was used to induce GP30 expression inside macrophages. The infection of THP-1 cells was performed using MS_gp30 and MS_Vec at an MOI of 10:1 and incubated for 4 h for phagocytosis. Cells were washed three times with sterile PBS, and then, hygromycin B (Roche) was added to a final concentration of 100 mg/mL to remove extracellular bacteria. At 4, 12, and 24 h after infection, the THP-1 cells were harvested, lysed with 0.025% SDS, serially diluted, and plated on 7H10 agar media with 20 µg/mL kanamycin. The plates were then cultivated at 37°C for four days until colonies could be counted.

Statistical analysis

All the data were analyzed with a two-tailed Student's t-test. Error bars represent standard deviation (SD). Statistical difference was defined as a *p*-value of 0.05.

Results

MS_gp30 constitutively expresses the GP30 protein

By using the NCBI Blastp server, GP30 protein was shown high sequence similarity to a trehalose-6,6'-dimycolate

(TDM)-binding peptide, PK34 (Fig. 1A), which has strong Mtb-killing activity (Wei *et al.*, 2013). The CASbig gp30 gene, which is 135 bp in size, encodes a 5 kDa peptide (Fig. 1B). To define the function of GP30, the recombinant strains MS_gp30 and MS_Vec were constructed. Both MS_Vec and MS_gp30 were cultured in Middlebrook 7H9 broth added 0.2% (w/v) glucose, 0.05% (v/v) Tween-80 and 0.2% (v/v) glycerol (pH 6.8, range 6.7–6.9). The molecular weight of the single myc tag is 1.2 kDa. Western blot analysis confirmed that an about 6.2 kDa Myc-GP30 fusion protein was detectable in the lysate of MS_gp30 using a mouse anti-Myc antibody, while it was absent in the control strain (MS_Vec) (Fig. 1C). These results reveal that GP30 from the mycobacteriophage CASbig was over-expressed in recombinant *M. smegmatis* mc² 155; therefore, the MS_gp30 recombinant strain could be used for subsequent study.

MS_gp30 is highly sensitive to acidic conditions

To evaluate whether GP30 has an effect similar to that of PK34 on the host, we next compared the growth pattern of MS_gp30

and MS_Vec in 7H9 broth (Fig. 2A) and minimal Sauton's media (Fig. 2B). As shown in Figs. 2A and 2B, these two recombinant bacteria presented the same growth pattern, indicating that over-expression of GP30 did not alter the growth kinetics of recombinant *M. smegmatis*. Phage-antibiotic synergy has been noted in multiple reports involving a variety of bacterial species (Comeau *et al.*, 2007; Kamal and Dennis, 2015; Matic *et al.*, 2016). To determine the effects of gp30 expression on the susceptibility of *M. smegmatis*, the minimal inhibitory concentrations (MICs) of various antimicrobial agents were analyzed by the broth dilution method (Liu and Nikaido, 1999; Ren and Liu, 2006). However, over-expression of gp30 gene did not bring change in the resistance of recombinant *M. smegmatis* to selected antibiotics significantly (data not shown). These results suggest that the GP30 protein has no role in the antibiotic susceptibility of *M. smegmatis*.

Interestingly, we found that MS_gp30 grew slower than MS_Vec when the mycobacteria were inoculated in acidic conditions (7H9 broth in Fig. 2C; Sauton's media in Fig. 2D), but no significant difference in media containing

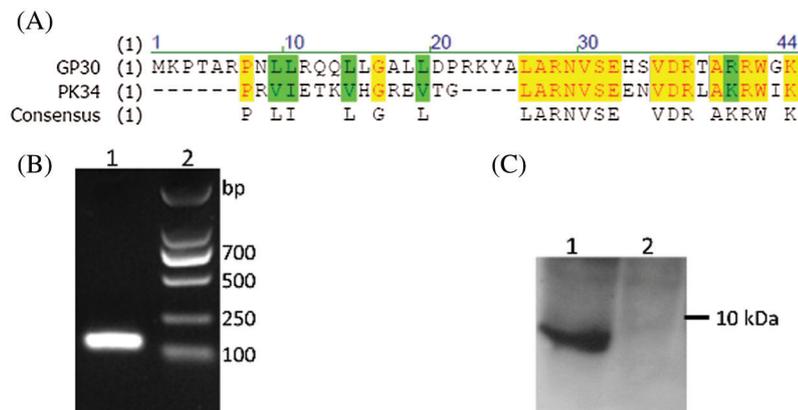


FIGURE 1. Heterologous expression of the gp30 gene in *M. smegmatis*.

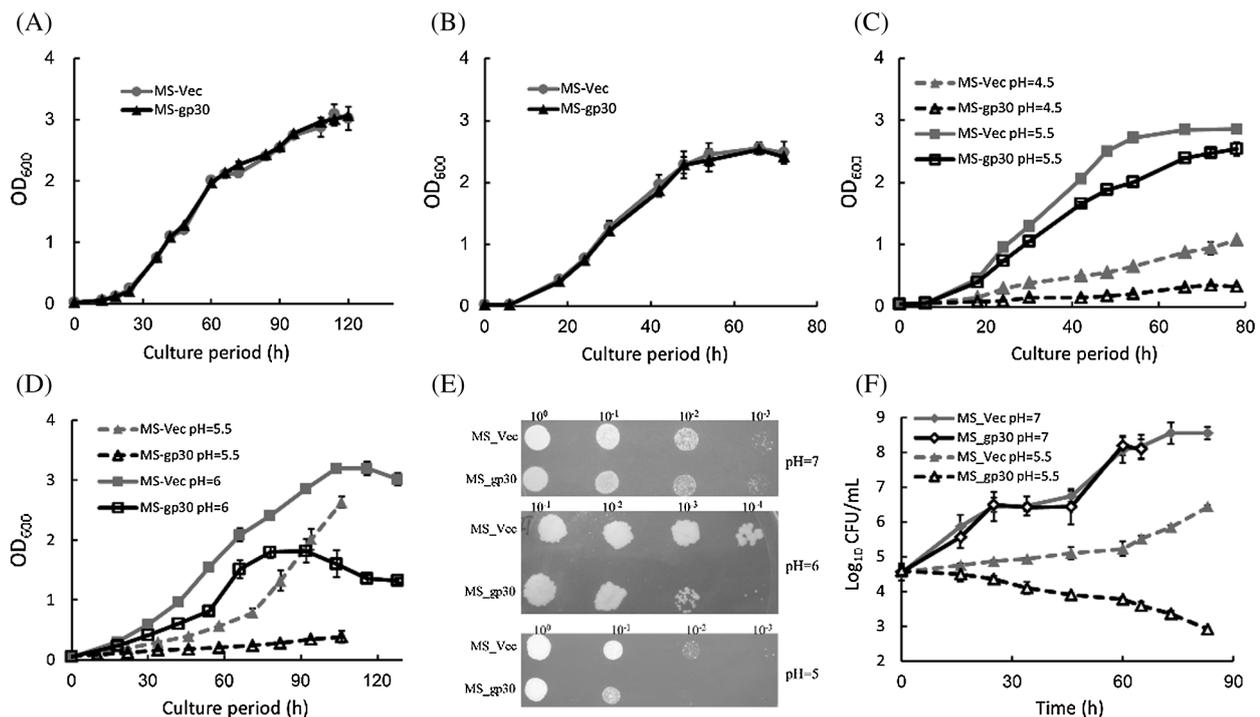


FIGURE 2. Survival of *M. smegmatis* expressing GP30 after challenge with acidic conditions.

the detergent SDS, lysozyme, or hydrogen peroxide (data not shown). We next evaluated the effect of GP30 expression on the resistance to acidic stress by comparing the survival rate of the recombinant MS_gp30 and MS_Vec after treated with low pH media for 6 h. These results seen in Fig. 2E suggest that MS_gp30 was more sensitive than MS_Vec in low pH media. As overexpression of GP30 increased susceptibility to acidic stress, we used acidic 7H9 broth (pH = 5.5) to perform a killing experiment; overexpression of gp30 caused greater susceptibility to the acidic broth and lower survival than those for in MS_Vec under the same treatment (Fig. 2F). In short, these results reveal that the GP30 protein negatively impacts mycobacterial acid adaptation.

GP30 alters the fatty acid profile and surface architecture of recombinant mycobacteria

To investigate whether the over-expression of GP30 in *M. smegmatis* can affect the lipid content, we evaluated the fatty acid profile of MS_gp30 and MS_Vec by GC-MS. We identified 26 major compounds in the two recombinant strains from C8-C24 (Fig. 3A), while MS_gp30 was found to have decreased total lipid content compared to those of the control (Fig. 3B). Several compounds were detected to be increased in MS_gp30, especially C8:0, C10:0, C11:0, C15:0, and C16:0, while others (C18:2N6C, C24:0, and C24:1) were diminished (Fig. 3C). Additionally, the cell diameter of the MS_Vec strain is normal at 0.3–0.4 μm , as shown in Fig. 3D. In contrast, the MS_gp30 strain was swelled by up to 1–1.15 μm in the cell diameter, as shown in Fig. 3E.

Taken together, these results suggest that GP30 can bring change in the lipid content and surface architecture of recombinant MS_gp30.

GP30 decreases the intracellular survival rate of recombinant MS_gp30

Since an intracellular acidic environment is a common strategy employed by macrophages to kill pathogens, we

wanted to explore whether GP30 can also reduce the intracellular survival rate of MS_gp30 within macrophages. In order to confirm this hypothesis, PMA-differentiated THP-1 cells were infected with MS_gp30 or MS_Vec at an MOI of 10. We compared the survival rate of MS_Vec and MS_gp30 in THP-1 cells. MS_gp30 showed significantly lower bacillary counts than the control strain in macrophages after 24 h infection (Fig. 4). This result suggests that the expression of GP30 impaired the intracellular survival rate of recombinant MS_gp30 inside human THP-1 macrophages.

Discussion

Here, we identified that a novel protein from the mycobacteriophage CASbig alters the surface architecture and fatty acid profile of the host. This study is the first to show that the mycobacteriophage CASbig protein GP30 is involved in mycobacterial acid adaptation and intracellular survival in macrophages.

To explore how GP30 influences mycobacterial physiology, the gp30 gene was expressed in the host *M. smegmatis* mc² 155. We detected that GP30 is involved in acid adaptation but not susceptibility to the other environmental stresses tested, including hydrogen peroxide, the detergent SDS and lysozyme. Mycobacteria are widely found and may be exposed to a great diversity of environmental conditions. *M. smegmatis*, a fast-growing saprophytic mycobacterium, generally grows over a broad range of pH, while *Mtb*, a representative slow-growing pathogenic mycobacterium, shows restricted growth in acidic pH (Paroha et al., 2017). Acidic conditions might be one of the most stressful environments to which bacteria may be exposed (Cosma et al., 2003). Humans are the only natural host for pathogenic mycobacteria, such as *Mtb*, while saprophytic mycobacteria, such as *M. smegmatis*, reside in water, soil, human skin, etc. (Paroha et al., 2017).

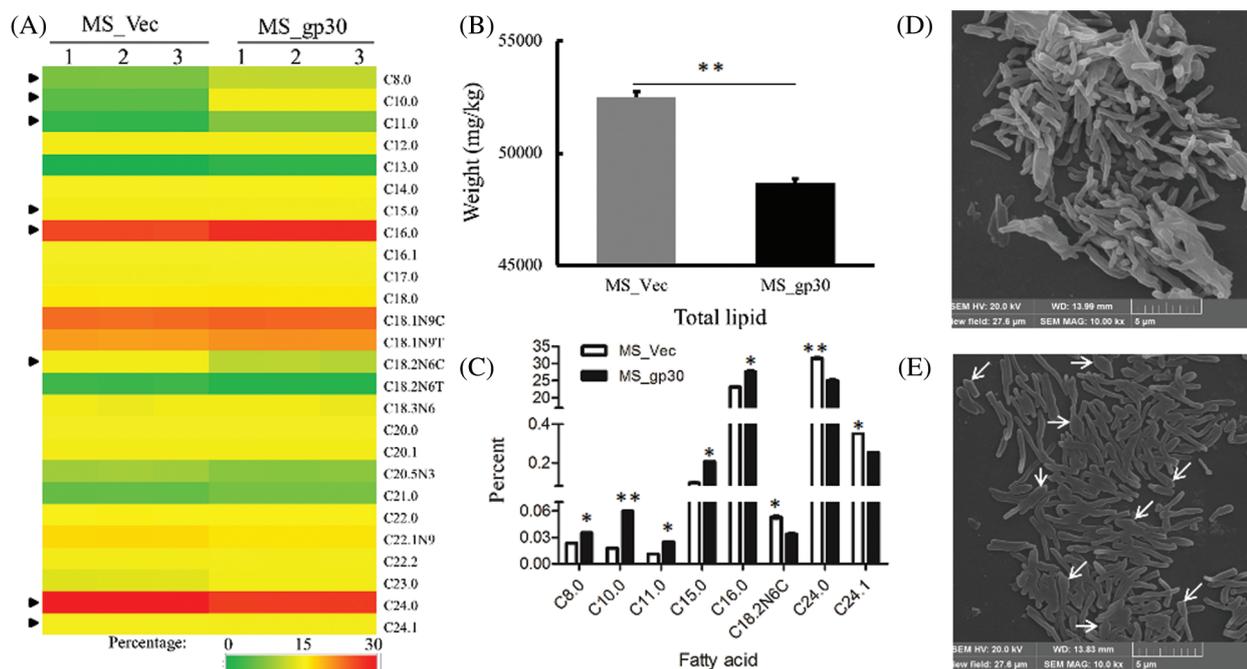


FIGURE 3. Analysis of the fatty acid profile and surface architecture of the recombinant *M. smegmatis* strains.

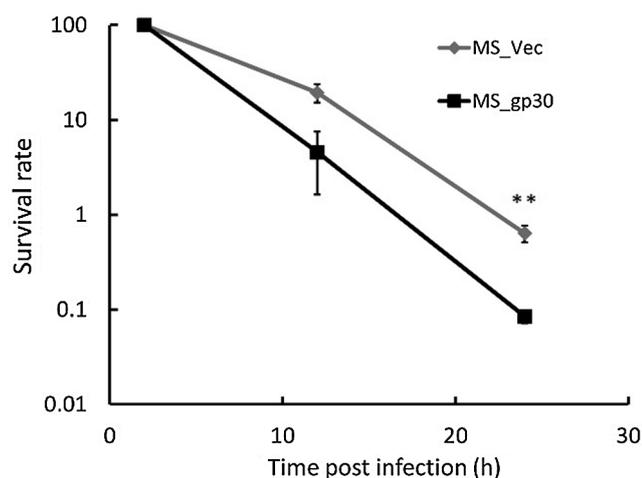


FIGURE 4. Intracellular survival of recombinant *M. smegmatis* in macrophages.

Sundaramurthy *et al.* (2017) showed that pathogenic mycobacteria can proliferate and reside in lysosomal compartments during the later stage of infection. These observations highlight that resistance to low pH is required for establishing infection. The specific role of GP30 during acid sensitivity is not known.

The mycobacterial cell wall is extremely complex and endows intrinsic resistance to stress conditions and drugs (Brennan, 2003; Shrimant *et al.*, 2018). Furthermore, Fozo and Quivey (2004) showed that *Streptococcus* mutants possess reduced levels of long-chained, monounsaturated fatty acids, and that the organism correspondingly becomes more acid-sensitive. Recently, we have also observed a similar phenomenon in recombinant protein-expressing *M. smegmatis* Rv2387 (Lv *et al.*, 2018). Thus, to explore the differences observed in acid adaptation of recombinant *M. smegmatis*, we examined the cell wall lipid composition and content of the MS_gp30 and MS_Vec strains by using GC-MS to extract and analyze the total lipids. The results are shown in Fig. 3B and reveal decreased total lipid accumulation in MS_gp30 compared to that in the control group. As shown in Fig. 3C, overexpression of GP30 led to a remarkable decrease in the percent of unsaturated, long-chain fatty acids (C18:2N6C, C24:0, and C24:1), which might decrease the resistance of MS_gp30 to low pH. Enhanced content of short-chain fatty acids in MS_gp30 compared to that in MS_Vec suggested that GP30 had affected the normal synthesis of long-chain fatty acids, the main components of the MS_gp30 cell wall. By blocking this ability, the organism becomes more acid-sensitive (Fozo and Quivey, 2004).

Here, we have identified a novel protein from mycobacteriophages involved in intracellular survival inside human macrophages. Although *M. smegmatis* is inherently considered nonpathogenic, it has been shown to behave in a pathogen-like manner by manipulating macrophages during the initial stages of infection, such as delaying phagosome acidification (Anes *et al.*, 2006; Kuehnel *et al.*, 2001). *M. smegmatis* has been broadly used as a surrogate bacterium to successfully characterize proteins implicated in intracellular survival (Li *et al.*, 2015; Li *et al.*, 2016; Pelosi

et al., 2012; Singh *et al.*, 2016; Wang *et al.*, 2015). The present study showed that MS_gp30 had significantly lower survival rate inside macrophage than the MS_Vec, suggesting a role in increasing the vulnerability of bacilli to low pH of the phagosome (Shrimant *et al.*, 2018; Vandal *et al.*, 2009). On the other hand, the decreased intracellular survival inside macrophages of MS_gp30 might be due to the protein's interference with the host immune response (Puiu and Julius, 2019), similar to the PK34 peptide (Wei *et al.*, 2013) and AK15 peptide (Yang *et al.*, 2019). Further research is warranted to explore the precise function of the GP30 protein during infection.

In summary, we found that expression of CASbig gp30 in the host *M. smegmatis* resulted in alteration of the surface architecture and fatty acid profile, ultimately impairing acid adaptation and intracellular survival in infected macrophages. The discovery of GP30 provides a novel antimycobacterial candidate and a novel approach to utilize phages.

Availability of Data and Materials: All data generated or analysed during this study are included in this published article.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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