



A simple multi-cloning strategy for gene expression and subcellular localization in mycobacteria

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ABSTRACT

Genetic manipulation of mycobacteria remains critical in functional genomics and modern microbiology. However, this process is often laborious due to limited cloning flexibility and low throughput. Here, we describe a streamlined cloning platform for expressing a gene of interest either alone or fused to a monomeric fluorescent protein under the control of two promoters of choice. This platform combines three simple PCR amplification reactions and four simultaneous ligations. Accordingly, this four-step procedure provides a fast and flexible cloning strategy for protein expression and subcellular localization analyses in mycobacteria. This cost-effective approach is compatible with some commonly used mycobacterial vectors and host strains. Using *M. tuberculosis* triacylglycerol synthase 1 gene (*tgs1*) as a proof of concept, we demonstrate the efficient cloning, robust expression, and reliable localization of the resulting fusion protein. This platform significantly reduces hands-on time and experimental complexity, providing a practical tool for functional and microbiological studies in mycobacteria.

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1. Introduction

Tuberculosis (TB), caused by its etiologic agent *Mycobacterium tuberculosis*, remains a significant global health issue with approximately 10.7 million cases and 1.23 million deaths in 2024 [1]. In this context, it is crucial to continue developing and implementing new strategies to better prevent, diagnose and treat TB worldwide.

In the quest of ending TB [1–3], basic research plays a key role in providing a deeper understanding of the molecular basis of

mycobacterial physiology, interaction with the host and susceptibility to antimicrobial compounds. Over the last two decades, genome-wide functional genomics have become a stepping stone that enable new discoveries by bridging the gap between the DNA sequence and the biological function of a specific gene [4–9]. Accordingly, many resources, datasets and technological tools have been developed for our community [9–13]. Despite the availability of these tools, genetic manipulation of mycobacterial species remains time-consuming and often laborious therefore constituting a strong bottleneck that slows down discoveries [14]. Thus, the development of simpler, cost effective and faster approaches that reduce hands-on time are constantly needed.

To overcome some of the limitations of traditional mycobacterial cloning methods, we have developed a series of vectors that enable expression of any gene of interest in its native form or as C-terminal fluorescent fusion, which can be controlled by two distinct promoters. This system has the potential to improve conventional restriction enzyme-based cloning by allowing a faster, simplified and cost-effective process. Our results clearly show that these vectors facilitate efficient gene expression, phenotypic investigation and subcellular localization in the model

Abbreviations: Anhydrotetracycline, (ATc); green fluorescent protein, (GFP); intrabacterial lipid inclusion, (ILI); triacylglycerol, (TAG); Tuberculosis, (TB); triacylglycerol synthase 1, (Tgs1).

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species *M. smegmatis*, providing a medium-throughput solution for functional genomic studies.

2. Material & Methods

2.1. Bacterial strains and culture conditions

M. smegmatis mc²155 and *M. tuberculosis* ATCC 25177 H37Ra reference strains were used in this study. All mycobacterial strains were routinely grown in Middlebrook 7H9 broth (BD Difco, #271310) supplemented with 0.2% glycerol (Euromedex, #EU3550) and 0.05% Tween 80 (Sigma-Aldrich, #P1754). In the case of *M. tuberculosis*, 10% oleic acid, albumin, dextrose and catalase (OADC enrichment; BD Difco, #211886) was also added. All cultures were maintained at 37 °C with shaking.

All molecular biology experiments including plasmid selection, expansion and maintenance were performed in the reference strain *Escherichia coli* DH10B (Invitrogen). *E. coli* was grown into LB Broth (BD Difco; #244620) or in LB solid medium containing Agar Agar Bacteriological (Euromedex; #1330). When required, hygromycin B (Toku-E, #H007) or kanamycin (Euromedex, #UK0010D) were used as selection markers for transformants on solid medium or for the culture maintenance of the recombinant strains. Final concentrations of 200 µg/mL and 50 µg/mL were used respectively for *E. coli* while 50 µg/mL was used for *M. smegmatis*.

2.2. Cloning and generation of pMSD vector series

All plasmids and primers used in this study are listed in supplementary information in Table S1 and S2. The constructs developed in this study derived from the well-established *E. coli*-*Mycobacteria* shuttle vector pUV15-pHGFP (Addgene plasmid #70045, kindly gifted by Sabine Ehrt) [15].

Briefly, the PtetO promoter was obtained by digesting the vector pOE1237 (kindly gifted by Tanya Parish) [16] with *SpeI* (New England Biolabs, #R3133L) and *PacI* (New England Biolabs, #R0547L), subsequently gel-purified (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel) and cloned at the *SpeI* and *PacI* sites of the digested pUV15-pHGFP to generate the vector pMSD1.

The TetR-coding sequence was amplified by PCR using Q5® high-fidelity polymerase (New England Biolabs, #M0491L) from the vector pJL32 [17] using the primers #P1 and #P2, subsequently gel-purified (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel) and cloned at the *SphI* (New England Biolabs, #R3182L) and *EcoRV* (New England Biolabs, #R3195S) sites of the digested pUV15-pHGFP to generate the vector pMSD32. Then, the TetR-coding sequence and its promoting region were amplified from pMSD32 using primers #P3 and #P4 and further cloned at the *NotI* (New England Biolabs, #R3189L) site of pMSD1 to generate the pMSD34.

mWasabi gene was amplified by PCR with the primers #P5 and #P7 by using the template vector pJL29 [17]. DNA fragments were gel-purified (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel) and further digested with *PacI* and *HindIII* restriction enzymes (New England Biolabs, #R0547L and #R3104L). Simultaneously, pUV15-pHGFP, pMSD1 and pMSD34 were digested with *PacI* and *HindIII* to remove the pH-GFP coding sequence. Purified and digested inserts and vectors were then ligated using T4 DNA ligase (New England Biolabs; #M0202L) to generate the pMSD24, the pMSD9 and pMSD40 vectors. Accordingly, pMSD24, pMSD9 and pMSD40 are fluorescent-based vectors that harbor the *mWasabi* gene under the control of the Psmc, PtetO without the TetR-coding sequence (*tetR*⁽⁻⁾) or PtetO with its corresponding TetR-coding sequence (*tetR*⁽⁺⁾).

Similarly, *mScarlet* gene was amplified by PCR with the primers #P10 and #12 by using the template vector pCas9-mScarlet (kindly

gifted by Qinglan Wang) [18]. DNA fragments were purified and further digested with *PacI* and *HindIII* restriction enzymes (New England Biolabs, #R0547L and #R3104L). Simultaneously, pUV15-pHGFP and pMSD34 were digested with *PacI* and *HindIII* to remove the pH-GFP coding sequence. Purified and digested inserts and vectors were then ligated using T4 DNA ligase (New England Biolabs; #M0202L) to generate the pMSD47 and pMSD48 vectors. Accordingly, pMSD47 and pMSD48 are fluorescent-based vectors that harbor the *mScarlet* gene under the control of the Psmc or PtetO with its corresponding TetR-coding sequence (*tetR*⁽⁺⁾).

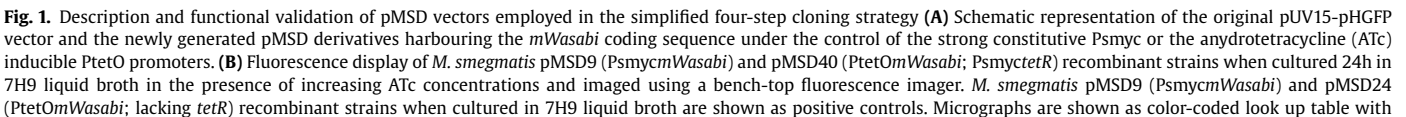
2.3. Principle, cloning procedure following this four-step procedure and proof of concept using *M. tuberculosis* *tgsl* (Rv3130c)

To generate vectors that harbor any gene of interest or their corresponding C-terminal fluorescent fusions, placed under the control of the constitutive Psmc or the anhydrotetracycline (ATc) inducible PtetO-TetR repression system, we designed a simple cloning strategy following a simple four-step procedure. As such, this process enables to target **one** gene of interest that can be placed under the control of **two** distinct promoters, by performing **three** PCR amplifications and **four** simultaneous ligations to rapidly clone and express the gene of interest under its native form or as a C-terminal fluorescent fusion.

Briefly, a target gene is amplified (PCR1) using a specific set of primers that include a 5' forward oligonucleotide that contains the *PacI* restriction site and a 6xHis tag coding sequence and a 3' reverse oligonucleotide that contains a restriction site producing 5'-CTAG overhangs compatible with *NheI* (*XbaI* in our case), a stop codon and a *HindIII* restriction site. In parallel, *mWasabi* (or alternatively *mScarlet*) is amplified (PCR2) using a specific set of primers that include a 5' forward oligonucleotide containing the *NheI* restriction site and a linker coding sequence in frame with the open reading frame of the fluorophore, and a 3' reverse oligonucleotide that contains a stop codon and a *HindIII* restriction site. Cloning of native forms is achieved by digesting the DNA product obtained from PCR1 by *PacI* and *HindIII* into the vectors pUV15-pHGFP or the pMSD34. Generation of *mWasabi* or *mScarlet* C-terminal fusions is achieved by digesting the DNA product obtained from PCR1 by *XbaI* (or alternative sites resulting in compatible ends with *NheI* such as *AvrII* or *SpeI*) which removes the stop codon and the *HindIII* restriction site, while the DNA product obtained in PCR2 is digested by *NheI*. Digested products are then ligated together using T4 DNA Ligase following manufacturer's instruction thanks to their compatible cohesive ends, and the newly ligated product further reamplified by PCR. This newly generated product (PCR3) is then digested by *PacI* and *HindIII* and subsequently cloned into the vectors pUV15-pHGFP and pMSD34.

As proof of concept, this process was carried out with the gene *tgsl* (Rv3130c) from *M. tuberculosis* and *mWasabi*. Hence, *tgsl* was PCR amplified from *M. tuberculosis* H37Ra reference strain genomic DNA using #P8 and #P9. Additionally, the *mWasabi*-coding sequence was PCR amplified from the pJL29 vector using #P6 and #P7. To construct *tgsl* expression vectors, the *tgsl* fragment was digested with *PacI* and *HindIII* and subsequently cloned within the pUV15-pHGFP or the pMSD34. This cloning procedure enabled to generate the pMSD39 and pMSD41 vectors respectively.

To create fluorescent fusions, *tgsl* PCR product was digested with *XbaI* (New England Biolabs; #R0145L) and *mWasabi* with *NheI* (New England Biolabs; #R3131L). Then, the two fragments were further ligated using T4 DNA ligase to generate a 2.1 kb DNA fragment, in which *tgsl* is fused in frame to *mWasabi*. This fragment was gel-purified (NucleoSpin® Gel and PCR Clean-up,



pixel intensities ranging from 0 to 255 units. (C) Fitted dose response analysis of normalized fluorescence intensity derived from *M. smegmatis* pMSD9 (PsmymWasabi) and pMSD40 (PtetOmWasabi; PsmymtetR) recombinant strains when cultured in 7H9 liquid broth in the presence of increasing ATc concentrations ranging from 0 ng/ml to 100 ng/ml for 24 h. (D) Schematic representation of the four-step strategy that enables the efficient cloning of any mycobacterial gene of interest in the pMSD series with mWasabi as potential fluorescent fusion.

Macherey-Nagel) and subsequently reamplified by PCR using #P8 and #P7, digested with *PacI* and *HindIII* and further cloned into pUV15-pHGF and pMSD34. Hence, the newly generated vectors pMSD37 and pMSD43 harbor the coding sequence of a 6xHis-tagged Tgs1-mWasabi fusion placed under the control Psmyc or PtetO-tetR⁽⁺⁾ repression system.

2.4. Mycobacterial transformation and selection

Preparation of competent mycobacterial cells and their subsequent electroporation was performed as previously described by Goude et al. [19]. Briefly, 100 mL of exponentially growing *M. smegmatis* mc²155 cells at an approximate optical density 600 nm (OD_{600nm}) of ~0.4–0.8 were washed 5 times at 4 °C in sterile aqueous solution containing 10% glycerol and 0.05% Tyloxapol (Sigma-Aldrich; #T8761). At each step, the volume washing solution was decreased (e.g., 50, 40, 30, 20 and 10 mL) and the final resuspension was performed in 1/50 to 1/100 of the original volume of culture. Then, approximately 500 ng to 1 µg of vectors of interest were added to 100–200 µL of competent cells and placed into an electroporation cuvette of 0.2 mm gap. Electroporation settings applied for *M. smegmatis* were 2.5 kV, 10 µF and 600 Ω. After electroporation, cells were transferred in 1 mL of complete 7H9 broth devoid of antibiotics and further incubated at 37 °C for 4 h before plating. Transformants were selected on complete Middlebrook 7H10 agar containing the appropriate concentration of hygromycin B and/or kanamycin. Plates were incubated at 37 °C for 4–5 days before recombinant clones were selected and further expanded in liquid cultures with the appropriate selection marker at a final concentration of 50 µg/mL.

2.5. Image-based and spectrofluorimetric monitoring of mWasabi levels

For fluorescence detection of mWasabi expressing vectors in 7H9 broth, recombinant bacteria harboring the plasmid pMSD24, pMSD9 or pMSD40 were dispensed into standard flat-bottom 96-well plates (Thermo Fisher Scientific; #167008) at an initial OD_{600nm} of 0.1 in the absence or presence of increasing concentration of anhydrotetracycline (ATc; Sigma-Aldrich; #37919) ranging from 0 to 200 ng/mL. At specific timepoints, the OD_{600nm} and the mWasabi-derived fluorescence signal were recorded using excitation/emission wavelengths of λ_{ex} 488 nm/λ_{em} 535 nm using a TECAN spark 10 M™ multimode microplate reader (Tecan Group Ltd, France). Fluorescent intensities were then normalized against OD_{600nm} of each well to ensure adequate normalization all along the kinetic. Data were analyzed and plotted using Graph Pad Prism 8.0.1 software. All the presented data have been obtained from experiments performed in technical triplicate at least on two to three distinct occasions.

Additionally, low-resolution imaging of liquid cultures of recombinant bacteria was performed as previously described [17] using the ChemiDoc™ MP Imaging System and the Image Lab software version 6.1.0 (Bio-Rad, France). Bright light acquisition was performed by using the 'White Epi Illumination' setting combined with the 'Standard Filter' and green fluorescence acquisition was performed by using the 'Blue Epi Illumination' setting combined with the '530/28 nm Filter'. When required, individual raw images were exported from Image Lab as TIFF files 600 dpi and displayed with the open-source software fiji/imagej

(<https://imagej.net/software/fiji/>) [20], to generate the final micrographs expressed as color-coded look up table where pixel intensities range from 0 to 255 units.

2.6. Whole-cell lysate preparation, immunoblotting and in-gel fluorescence assay

Recombinant *M. smegmatis* mc²155 harboring pMSD24 and pMSD37 were grown in complete 7H9 medium supplemented with 50 µg/mL of hygromycin when necessary, and incubated at 37 °C under shaking at 180–200 rpm until OD_{600nm} reached late exponentially growing phase (approximately OD_{600nm} 1.0–2.0). At indicated timepoints, OD_{600nm} was monitored and subsequently used to normalize each bacterial culture. Normalized samples then were harvested for 10 min at 4000×g, then washed twice with PBS (pH 7.4) buffer containing 0.05% (v/v) Tween-20, and re-suspended with PBS (pH 7.4) buffer. Then, bacterial cells were mixed with 200 µL of 0.1 mm diameter glass beads (BioSpec), and mechanically disrupted during 3 × 4 min of vigorous shaking using Mini-Beadbeater-96 (BioSpec, USA). The lysates were centrifuged for 5 min at 1500 rpm and the resulting supernatant (~750 µL) was carefully collected. Samples were diluted in Laemmli Sample Buffer, incubated at 95 °C for 5–10 min and stored at –20 °C. Each sample was then loaded and separated on to 12% SDS/PAGE and further transferred on to a 0.2 µm PVDF membrane (Bio-Rad, #1704156) using a Trans-Blot Turbo Transfer System (Bio-Rad). Immunoblotting of 6×His-tagged proteins was performed using anti-HisProbe™ HRP conjugate according to the manufacturer's instructions (Thermo Fisher Scientific, #15165). Coomassie blue/Ponceau Red Staining and/or detection of GroEL1 levels through its natural poly-histidine sequence present in its C-terminal domain were used as internal loading controls. Detection was achieved using Pierce™ ECL Western Blotting substrate solution (Thermo Fisher Scientific, #32132) and visualized with a ChemiDoc™ MP Imaging System (Bio-Rad). For in-gel fluorescence assay (Fig. S2), normalized whole-cell lysates were diluted in Laemmli Sample Buffer, without incubation at 95 °C, then loaded and separated on to 12% SDS/PAGE. Coomassie blue staining was used as loading control. Detection mWasabi and Tgs1-mWasabi signal was performed with a ChemiDoc™ MP Imaging System (Bio-Rad) by applying the green fluorescence acquisition settings using the 'Blue Epi Illumination' setting combined with the '530/28 nm Filter'.

2.7. Subcellular localization of *M. tuberculosis* Tgs1 using pMSD vectors and fluorescence microscopy

M. smegmatis WT and *M. smegmatis* pMSD24 and pMSD37 recombinant strains were grown in Middlebrook 7H9 broth supplemented with 5% glycerol (Euromedex, #EU3550) and 0.05% Tween 80 (Sigma-Aldrich, #P1754) to promote TAG formation [21]. After 24h, 1 mL of bacterial cultures were harvested 5 min at 5000 g, subsequently washed twice with PBS buffer and stained with Nile Red (Interchim, FluorProbes, #FP-46875A) as previously described [21]. Briefly, 5 µL (0.5 mg/mL in absolute ethanol) of Nile Red fluorescent dye was added to 100 µL of cell suspension. Cells were further incubated for 20–30 min at 37 °C in the dark. Stained bacteria were harvested for 5 min at 5000 g, then washed twice with 500 µL of PBS-0.05% Tween 80 buffer and finally re-suspended in 300 µL of PBS. Each bacterial suspension (5 µL) was

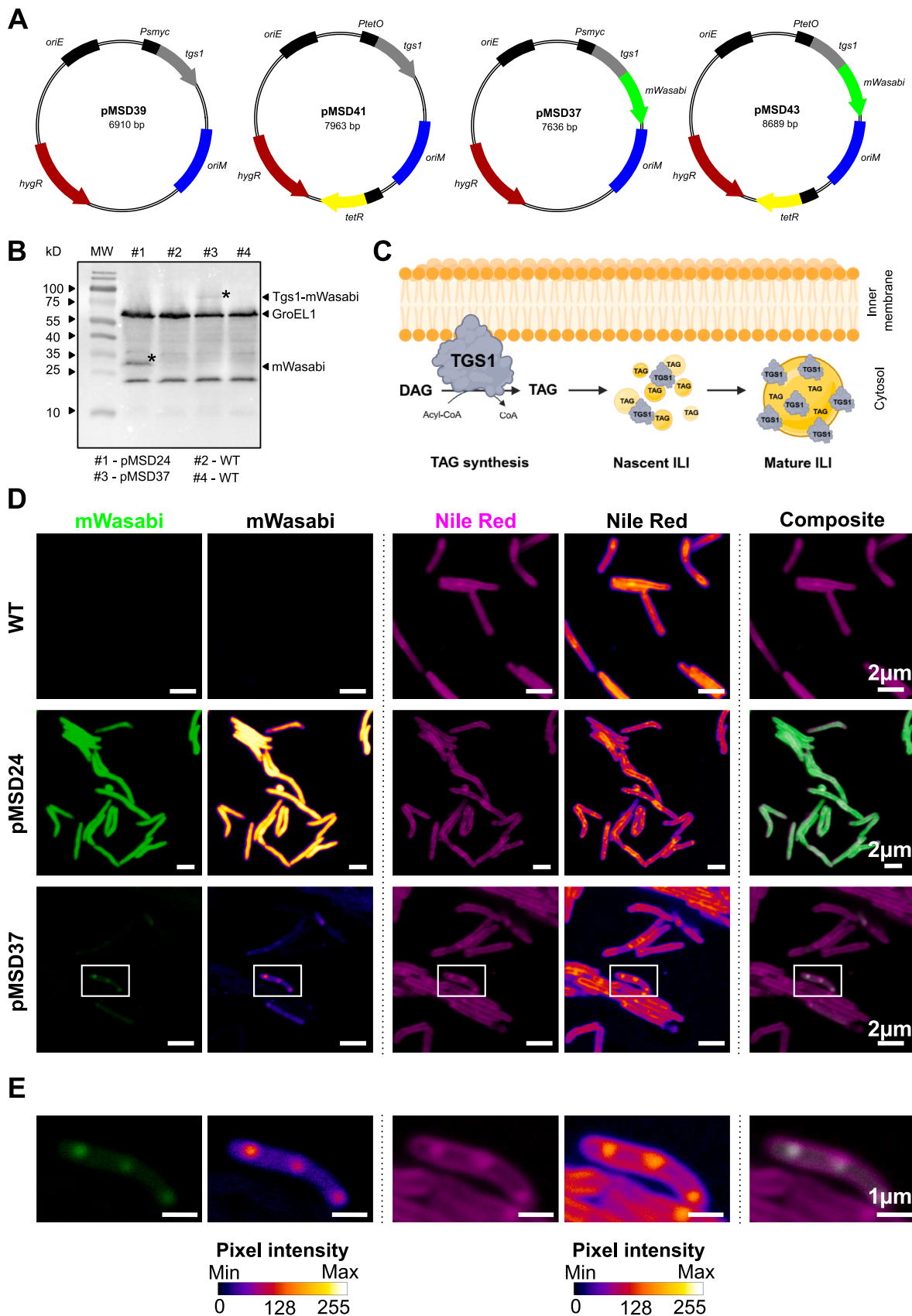


Fig. 2. Phenotypic validation and spatial localization of Tgs1 in mycobacteria using vectors from the pMSD series. (A) Schematic representation of the pMSD derivatives harbouring the *tgs1* or the *tgs1*-mWasabi-coding sequences under the control of the strong constitutive Psmyc or the anhydrotetracycline (Atc) inducible PtetO promoters. (B) Immunoblot analysis of 6xHis tagged protein levels in *M. smegmatis* mc² 155, highlighting mWasabi and Tgs1-mWasabi production. (C) Schematic representation of Tgs1 function in TAG production in *M. tuberculosis*. (D) Tgs1-mWasabi localizes with Nile Red positive-ILI under carbon rich conditions. Fluorescence imaging of live *M. smegmatis* WT or

recombinant strains producing mWasabi alone or Tgs1-mWasabi stained with the neutral lipid dye Nile Red. Samples were imaged in both green fluorescent channel (top panel; shown in green and in grayscale as look up table) and the red fluorescent channel (bottom panel; shown in magenta and in grayscale as look up table). A merged micrograph is also displayed with a representative drawing of the colocalization. Each individual channel is also represented as 16-colors scaled images on the right (0-255 grayscale units as look up table) to highlight Nile Red specific structures such as ILI and mWasabi distribution profiles. Scale bars represent 2 μm in both X and Y dimensions. (E) High magnification views of the zoomed-in regions from the images displayed in (D) are shown where scale bar represents 1 μm in both X and Y dimensions.

spotted between a coverslip of 170 μm thickness and a freshly prepared 1.5% agarose-PBS pad of approximately 1–2 cm^2 . Bacteria were analyzed by snapshot imaging at room temperature using a Leica SP5 laser scanning confocal microscope (Leica Biosystems, Nussloch, Germany) as previously described [17,22]. Image acquisition was performed with an HC PL APO CS2 63 $\times$ /1.40 oil objective. Images of 1024 $\times$ 1024 pixels were acquired with argon 488 nm and diode-pumped solid-state 561 nm lasers. Emitted signal was collected at λ_{em} 500–550 nm and λ_{em} 585–650 nm for mWasabi and Nile Red channels, respectively. One single Z-plane was acquired for each field, and a minimum of 5 fields per biological sample were imaged. Individual raw fluorescence images were exported as TIFF files and displayed with open-source software such as Fiji/ImageJ software (<https://imagej.net/software/fiji/>) [20]. Visualization was performed at least on two independent occasions.

3. Results & discussion

We recently capitalized on the well-established mycobacterial-*E. coli* shuttle vector pUV15-pHGF [15] (Fig. 1A) to construct a new subset of vectors harboring green or red fluorophores-coding sequences under the control of the strong and constitutive Psmc promoter [17,23]. Mycobacterial species such as *M. smegmatis* or *M. tuberculosis* transformed with pJL29 and pJL30 vectors displayed very bright fluorescent signals at multiple scales from colonies to single-cells suggesting these vectors and their respective fluorophores might represent interesting tools to investigate the spatial distribution of proteins in different mycobacterial species by microscopy [17]. Hence, we generated a new series of vectors (Fig. 1A) that enable the expression of a 6xHis-tagged version of the bright and monomeric mWasabi or mScarlet proteins respectively, under the control of the strong and constitutive Psmc or the PtetO operator (Fig. 1A, Fig. S1A). The latter tool was generated in the absence or presence of its corresponding repressor TetR, to finely tune expression levels using the well-characterized anhydrotetracycline (ATc) inducible TetR/PtetO repression system (Fig. 1A) [23,24].

Functional validation of these vectors in the reference strain *M. smegmatis* mc²155 demonstrated that pMSD24 and pMSD9, harboring Psmc or PtetO/TetR⁽⁻⁾, respectively, displayed strong mWasabi fluorescent signals that remained stable even after 24 h of incubation (Fig. 1B). Comparative analysis of *M. smegmatis* cells containing pMSD9 and pMSD40, which harbor PtetO/TetR⁽⁻⁾ or PtetO/TetR⁽⁺⁾ repression systems, confirmed that the introduction of TetR enables to tightly repress mWasabi expression (Fig. 1B). Dose-dependent analysis of ATc-mediated derepression, showed that ATc treatment led to a rapid, dose-dependent mWasabi signal emission (Fig. 1C). This fully confirms that these tools enable a chemically-controlled and tunable expression of mWasabi in mycobacteria.

We then designed and used a simple four-step cloning procedure to virtually produce any native or mWasabi/mScarlet-fused protein of interest to facilitate the phenotypic investigation of their biological function and the characterization of their spatiotemporal localization by fluorescence microscopy [25]. Hence, this process enables to target one gene of interest that can be placed under the control of two distinct promoters, by performing three PCR amplifications and four simultaneous ligations to

rapidly clone and express the gene of interest under its native form or as a C-terminal fluorescent fusion. A schematic representation of this four-step cloning process is shown in Fig. 1D and Fig. S1B, and fully described in the Material & Methods section.

As proof of concept, we capitalized from this approach to express within *M. smegmatis*, *M. tuberculosis* tgs1 (Rv3130c) which encodes the major triacylglycerol synthase in the tubercle bacilli [26,27]. Thus, we constructed the vector pMSD37 which harbor the coding sequence of a 6xHis-tagged Tgs1-mWasabi fusion placed under the control of the strong promoter Psmc (Fig. 2A). Production of recombinant proteins was assessed by microscopy and by immunoblotting of the 6xHis tag using conventional anti-His HRP conjugated antibody. Immunoblot experiments confirmed the production of Tgs1-mWasabi fusion (Fig. 2B). Indeed, with an apparent molecular weight of 80kD, we observed that the fluorescent fusion is well-produced. A positive band around 55kD corresponding to GroEL1 which possesses a natural 5xHis-tail in C-terminal position [28], was also detected and could serve as an additional normalization control (Fig. 2B). In addition to this immunoblot, we also performed in-gel mWasabi fluorescence visualization by performing conventional SDS-PAGE within heating *M. smegmatis* protein lysates. Results revealed the production of mWasabi and Tgs1-mWasabi under its full-length (Fig. S2A and S2B). Nevertheless, a partial proteolytic cleavage of the fluorescent fusion was also observed resulting in a sub-fraction of free mWasabi (Fig. S2A and S2B). This result suggests that the well-established linker used by Zhu et al. [25] might be subjected to mycobacterial proteolytic activities, and therefore alternative linker might be more suitable to enhance fusion stability. Taking into account this observation, this strain was used for subsequent localization studies.

Since, *M. tuberculosis* tgs1 (Rv3130c) is known to catalyze TAG biosynthesis first at the inner leaflet of the cytosolic membrane and to further relocate to mature intrabacterial lipid inclusion (ILI) (Fig. 2C) [26,27,29–31], we sought to demonstrate that our vectors can be used to monitor the spatial distribution of this protein of interest by live snap-shot fluorescence microscopy (Fig. 2D). Thus, recombinant bacteria expressing mWasabi alone or the Tgs1-mWasabi fusion were grown in carbon rich medium and further stained with the neutral lipid dye Nile Red. Images show that when produced under its native form, mWasabi displays, as expected, a bright and diffuse signal within the bacterial cytoplasm (Fig. 2D). In contrast, the Tgs1-mWasabi fusion appears to form of small foci that colocalize with both membrane-associated or cytosolic Nile Red positive structures. These are likely to correspond to early events of TAG-containing inclusion biogenesis and the formation of more mature ILI, as previously reported (Fig. 2D & E) [29–31]. Accordingly, these results provide a strong proof of concept that the pMSD vector series can be used to investigate the biological function and spatiotemporal localization of a given mycobacterial protein.

4. Conclusions

In this Short communication, we describe the generation of the pMSD series, a new subset of *E. coli*-Mycobacteria shuttle vectors for mycobacterial research. We provide a simple, efficient and cost-effective process to amplify and clone any gene of interest into these vectors, to express these genes as native or fluorescently-fused form under the control of different promoters.

This approach usually takes a standard week of working days to generate a complete set of Psmc and PtetO-driven constructs, containing the gene of interest alone or fused to a C-terminal fluorescent fusion such as mWasabi or mScarlet. By replacing the conventional overlapping PCR procedure that physically links two or three fragments together during amplification process, we reduced hands-on time and troubleshoot related to successful amplification of high-GC content DNA products. Using an alternative restriction site, has proven to be simple and effective making this technique extremely cost-effective. More importantly, under its current design, it considerably reduced the number of primers required to generate these distinct constructs. Several alternatives to these two cloning approaches could be of interest to reduce further cloning time-frame. As such, implementing seamless cloning based on In-fusion®, Golden-Gate or NEBuilder® HiFi technology could be worth investigating. It is worth mentioning that during the preparation of our manuscript, another plasmid toolbox has been described [32], using alternative vector backbones and cloning approaches. While the two studies report simplified cloning procedures and localization studies, we believe the two studies are perfectly complementary. Finally, we provide a solid proof of concept by applying this process to the well-established *tgsl* gene of *M. tuberculosis* which is key in regulating TAG levels by interacting with nascent and mature ILI. Overall, we anticipate that making these tools available to our community, might have the potential to advance TB research by deciphering the biological function and spatio-temporal localization of proteins in mycobacteria.

CRedit authorship contribution statement

Maria Sofia Deschutter: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition. **Eva Beaumale:** Writing – original draft, Visualization, Methodology, Investigation. **Stéphane Canaan:** Writing – original draft, Supervision, Funding acquisition. **Gabriela Gago:** Writing – original draft, Supervision, Funding acquisition. **Pierre Santucci:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Data accessibility

Plasmid vectors from the pMSD series and any additional data that support the findings of this study are available upon reasonable request from the corresponding authors at deschutter@ibr-conicet.gov.ar; gago@ibr-conicet.gov.ar or psantucci@imm.cnrs.fr.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used <https://chat.mistral.ai/> in order to perform some spell check, identify grammatical errors and improve the style and tone of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of this article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biochi.2026.08.008>.

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