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Green Fluorescent Protein as a protein localization and topological reporter in mycobacteria

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ABSTRACT

The cell envelope-associated proteins of *Mycobacterium* species play critical functions in the physiology and pathogenicity of these microorganisms. Because the determination of their subcellular localization and transmembrane topology is often critical to the understanding of their function, we investigated whether the Green Fluorescent Protein (GFP) could be used as a reporter to probe protein localization and map the topology of inner membrane proteins directly in intact mycobacterial cells. To this end, two GFP-based mycobacterial reporter plasmids were engineered and their functionality validated using a variety of membrane-associated, exported and cytosolic proteins.

The secreted and cell envelope-associated proteins of *Mycobacterium tuberculosis* play critical roles in the pathogenicity of and generation of protective immunity to the bacterium and have proven invaluable from the perspective of the development of novel vaccines and diagnostics [1]. They also play an important role in the physiology of the bacterium, participating in numerous enzymatic and transport functions such as nutrient import, the building of the mycobacterial cell envelope, cell elongation and cell division and, as such, they represent attractive targets for the development of novel therapeutic agents.

Determining the subcellular localization and topology of cell envelope-associated proteins is critical to the understanding of their biological activities and physiological functions. To this end, reporter fusions with *lacZ*, *phoA* and *blaC* have been extensively used to differentiate exported proteins from cytosolic ones and map the topology of inner membrane proteins [2–5]. *phoA* encodes an alkaline phosphatase that folds into an enzymatically active conformation only in the periplasm. β-galactosidase (β-Gal), encoded by *lacZ*, functions in the opposite manner of PhoA in that it is enzymatically active only when retained in the cytoplasm. BlaC is a β-lactamase whose export to the periplasm protects mycobacteria from the effect of β-lactam antibiotics [5]. While functional in mycobacteria, limitations of these reporter genes include the requirement for sample lysis and specific substrates to obtain measurement, the need to work in the background of β-lactam-susceptible mutants devoid of endogenous β-lactamase activity in the case of BlaC, and the reported toxicity of some exported β-Gal fusions [6]. Moreover, because *phoA* and *lacZ* reporter systems are more widespread in *E. coli* than in mycobacteria, *E. coli* is often used as a surrogate host to express mycobacterial gene fusions, with the caveat that the information yielded by these heterologously expressed fusions may not accurately reflect the subcellular location of the native protein domains produced in mycobacteria.

The Green Fluorescent Protein from *Aequorea victoria* (GFP) is a relatively small (27 kDa) protein that stably fluoresces as a monomer. Its stability, ease of detection in intact cells and low detection limits (<10 ng of GFP per ml of culture; 5 ng of GFP per protein band on a standard SDS-PAGE gel) have made it a reporter of choice to probe a variety of events within living eukaryotic and prokaryotic cells. The fact that its activity can be detected directly in intact cells without requiring any specific substrates eliminates the need for time-consuming protein extraction, enzymatic reactions and immunoblots. For this reason, GFP has been used extensively to directly monitor the level of expression, folding, and stability of proteins (particularly inner membrane proteins) throughout their production, solubilization and purification from *E. coli* [7] and yeast cells [8]. The finding that GFP folds efficiently in the cytoplasm of *E. coli* but does not form an active enzyme when targeted to the

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periplasm [6] has further prompted applications of this protein as a transmembrane topological marker [9]. Despite its convenience as an expression and topological reporter, the use of GFP and other fluorescent proteins in mycobacteria have thus far essentially been restricted to the screening of compounds interfering with the replication of *M. tuberculosis in vitro* and inside macrophages or to monitoring gene expression and interactions between mycobacteria and host cells. Few examples exist wherein GFP was used to determine protein localization and this marker has, to the best of our knowledge, never been used to map the topology of inner membrane proteins directly in mycobacteria. The primary goal of this study was to determine whether GFP could be used as a reporter to probe the subcellular localization of proteins as well as the transmembrane topology of inner membrane proteins in intact mycobacterial cells. Two convenient expression plasmids were engineered to this end, and tested on a panel of mycobacterial transmembrane, exported and cytosolic proteins.

The pJB vector system consists of two vectors, pJB (−) and JB (+), engineered from the mycobacterial expression plasmid, pMV261 [10], and the *E. coli* expression plasmids, pWARF (−) or pWARF (+) [11]. This system allows for the mycobacterial expression under control of the p*hsp60* promoter of proteins C-terminally-fused to the GFP and to a octahistidine tag that may subsequently be easily purified from a *Mycobacterium* host. pJB (−) has the HRV 3C protease recognition site (LEVLFQ/GP), followed by GFP and a C-terminal His_8 tag. pJB (+) has the HRV 3C protease cleavage site, followed by the transmembrane segment of glycophorin A, GFP and a C-terminal His_8 tag [Fig. 1A] [11]. Gene fusions in pJB (−) and JB (+) may be generated from the same PCR fragment. Based on earlier *E. coli* studies [11], it was expected that the addition of a single transmembrane domain from glycophorin A between the C-terminal fusion point of the protein of interest and the GFP in pJB (+) would allow membrane proteins with extracellular C-terminal fusions in mycobacteria to be converted to proteins with intracellular C-terminal fusions [Fig. 1B]. Because GFP fluoresces in the cytoplasm but not in the periplasm, a high fluorescence signal in the pJB (−) version and background fluorescence in the pJB (+) version are indicative of the C-terminal fusion of the protein being cytoplasmic. Opposite fluorescence intensities indicate, on the contrary, that the C-terminal fusion of the protein is periplasmic.

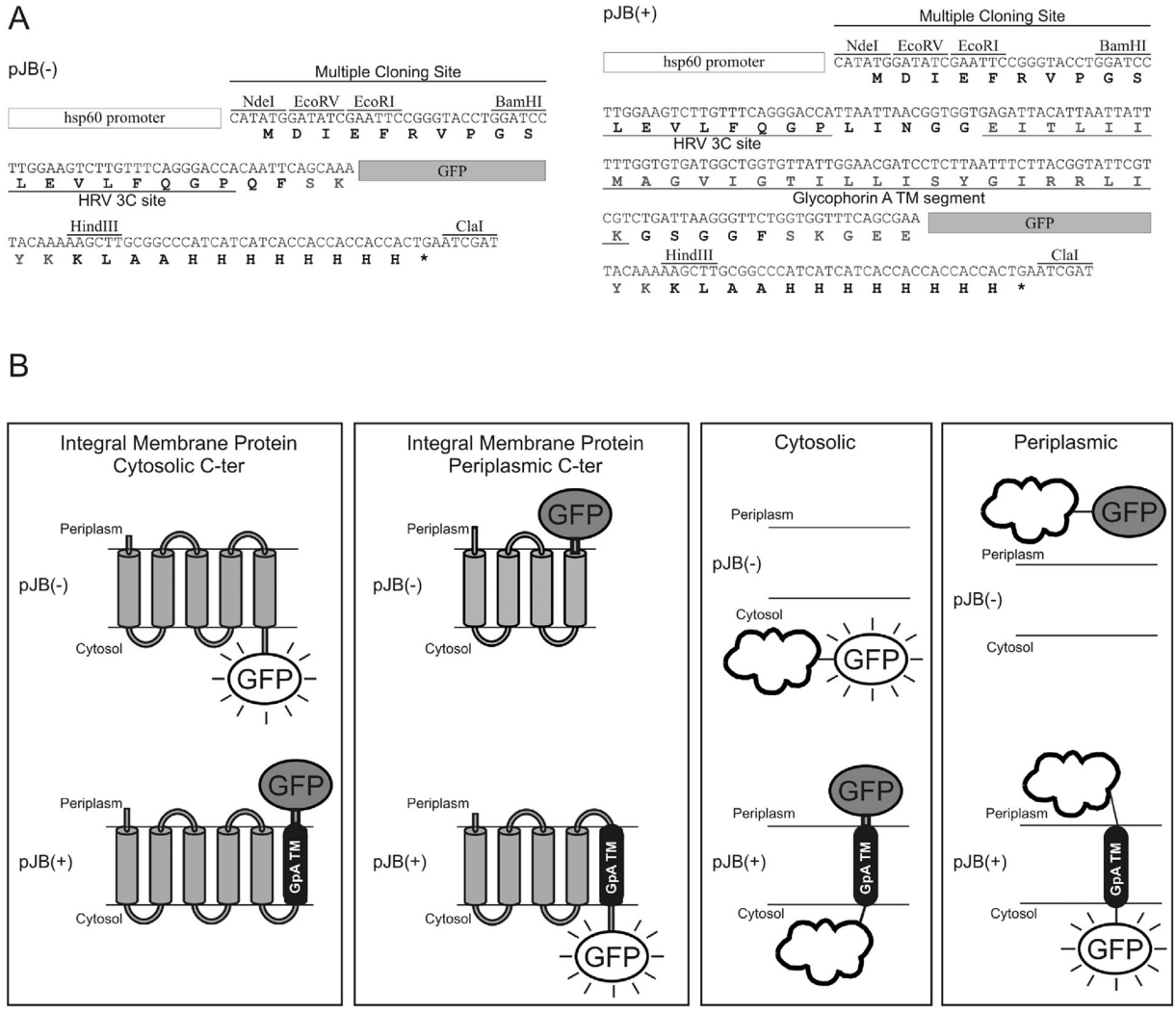

Fig. 1. The pJB (−) and pJB (+) expression plasmids and principle of the GFP fusions generated in this reporter system. **(A)** Relevant features of the pJB (−) and pJB (+) plasmids showing the localization of the *hsp60* promoter, the multiple cloning sites, the region encoding the transmembrane (TM) segment of glycophorin A (GpA), and the *gfp* gene. **(B)** Principle of the GFP reporter fusions generated in the pJB (−)/pJB (+) system. pJB (−) fuses GFP to the C-terminus of a protein. GFP fluoresces if the C-terminus is in the cytoplasm. pJB (+) fuses glycophorin A and GFP to the C-terminus of a protein. Periplasmically-oriented C-termini from Sec-dependent inner membrane and exported/secreted proteins are reoriented to the cytoplasmic side of the inner membrane by the single transmembrane domain of glycophorin A leading to GFP fluorescence.