Monitoring promoter activity and protein localization in *Mycobacterium* spp. using green fluorescent protein

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Abstract

Two green fluorescent protein (Gfp) fusion vectors were constructed for use in *Mycobacterium* spp. The first plasmid facilitates quantification of mycobacterial promoter activity. The second vector permits construction of translational fusions of mycobacterial proteins to Gfp in order to study subcellular localization including protein secretion. Using this translational fusion construct, we verify that a Gfp fusion to the putative secreted *M. tuberculosis* protein ChoD is translocated to the extracellular milieu when cloned and expressed in *Mycobacterium smegmatis*. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The green fluorescent protein (Gfp) has become a popular reporter system for use in both prokaryotes and eukaryotes. In eukaryotes, Gfp is used frequently for numerous applications, including transcriptional fusions to study gene expression, as well as translational protein fusions to study protein targeting (reviewed in Tsien, 1998). The study of Gfp fusion protein targeting in eukaryotes has been used to identify proteins targeted to cellular locations such as the plasma membrane (Yokoe and Meyer, 1996), the endoplasmic reticulum (Miyawaki et al., 1997), and the phagosome (Maniak et al., 1995). In prokaryotes, the *gfp* gene has been used primarily as a reporter of promoter activity by creating transcriptional fusions in a wide variety of bacterial species, including *Brucella suis* (Kohler et al., 1999), *Salmonella typhimurium* (Valdivia et al., 1996), and *Mycobacterium* species (Valdivia et al., 1996; Kremer et al., 1995; Dhandayuthapani et al., 1995). Indeed, the list of Gfp transcrip-

tional fusion vectors for use in mycobacteria has grown in the last 5 years, and several studies have demonstrated that Gfp is extremely useful as a method for monitoring gene expression in mycobacteria, including localization of the organism within the host macrophage (Dhandayuthapani et al., 1995; Parker and Bermudez, 1997; Barker et al., 1998; Via et al., 1998a,b; Luo et al., 1996; Teitelbaum et al., 1999).

In contrast, the use of chimeric *gfp*-prokaryotic protein fusions in bacteria is in its early development as a tool to study protein targeting. It has recently been shown to be possible to identify the intracellular location of GFP fusion proteins within bacteria using confocal microscopy. The list of such proteins includes the localization of MinE and FtsZ of *E. coli* to the cell septum during cell division and localization and monitoring of the expression of the *Streptomyces clavuligerus* protein LAT during colony growth (Ma et al., 1996; Raskin and de Boer, 1997; Khetan et al., 2000). Furthermore, a recent report has used protein fusions between mycobacterial antigens and Gfp to monitor protein expression in a DNA vaccine in murine fibroblasts (Velaz-Faircloth et al., 1999), thus demonstrating one of the potential uses for Gfp protein fusions in monitoring bacterial protein localization in eukaryotic cells. However, perhaps due to the low levels of expression of Mycobacterial promoters, the translational fusion approach has not been used for protein localization in mycobacteria.

A system for studying targeting of *M. tuberculosis*

proteins both within the organism itself and within the host macrophage during infection would be a useful tool for the identification of proteins secreted by *M. tuberculosis*. The exact intracellular compartment to which candidate proteins are targeted within the macrophage may be identified using confocal fluorescence microscopy. In this study, we constructed a set of *E. coli- Mycobacterium* shuttle vectors to be used for the creation and analysis of transcriptional and translational Gfp fusions in *Mycobacterium* species. We demonstrate that these vectors are functional in *Mycobacterium smegmatis* and *M. bovis* BCG, and that through the use of a highly active *M. tuberculosis* promoter, it is possible to identify secreted mycobacterial Gfp fusion proteins.

2. Materials and methods

2.1. Strains and plasmids

The bacterial strains, plasmids, and oligonucleotides used in this study are given in Table 1. *Escherichia coli* was grown in Luria–Bertani medium at 37°C. Ampicillin (100 µg/ml) and hygromycin (150 µg/ml) were used whenever required. *Mycobacterium smegmatis* mc^{2}155 and *M. bovis* BCG were grown in 7H9 medium with 10% OADC and 0.05% Tween-20. Hygromycin (50 µg/ml) was used when required. The oligonucleotides, shown in Table 1, were synthesized by Gibco-BRL Laboratories.

2.2. Construction of E. coli- Mycobacterium transcriptional and translational Gfp fusion shuttle vectors

The *E. coli-Mycobacterium* green fluorescent protein transcriptional fusion vector pSC301 was constructed as follows: the enhanced Gfp cassette was excised from pGreenTIR (Miller and Lindow, 1997) using *Eco*RI, Vent-polymerase blunt-ended, and ligated into the *Hind*III site of pSODIT (De Smet et al., 1999) that had been similarly blunt-ended. The resulting Gfp transcriptional fusion vector was designed to contain an ATG translational start codon and a consensus Shine-Dalgarno with a spacer region that minimizes RNA secondary structure (Fig. 1A). This Gfp cassette is located downstream of a multiple cloning site and the *M. tuberculosis* superoxide dismutase (SOD) promoter.

The *E. coli-Mycobacterium* green fluorescent protein translational fusion vector pSC300 was constructed as follows: the enhanced Gfp gene was excised from the plasmid pGreen (Miller and Lindow, 1997) using *Acc*55I, subsequently Vent-polymerase blunt-ended, and cloned into the *Hind*III site of pSODIT (Ainsa et al., 1998) that had been similarly blunt-ended. This Gfp gene lacks the signals for translation initiation, and starts with the second codon for enhanced Gfp (Fig. 2A).

2.3. Cloning of transcriptional and translational fusion constructs

The *pkn*H transcriptional fusion was made by PCR amplification of a sequence that includes 17 nucleotides past the

Table 1
Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Characteristics[a]	Source or reference
Strains		
E. coli DH5α	F$^-$ *rec*A1 *hsd*R17 *thi*-1 *gyr*A96 *sup*E44 *end*A1 *rel*A1 *rec*A1 *deo*R Δ(*lac*ZYA-*arg*F)U169 (φ80 *lac*Z ΔM15)	
M. smegmatis	mc^{2}155	W.R. Jacobs
M. bovis BCG	Pasteur strain	ATCC 35374
Plasmids		
pCR2.1	TA cloning vector, Amp^r	Invitrogen
PSODIT	*E. coli - Mycobacterium* shuttle plasmid, Hyg^r	De Smet et al., 1999
Pgreen	Enhanced Gfp cassette plasmid, Amp^r	Miller and Lindow, 1997
PgreenTIR	Enhanced Gfp cassette plasmid containing transcriptional enhancer region (TIR) upstream of *gfp*, Amp^r	Miller and Lindow, 1997
pSC300	pSODIT containing *gfp* from pGreen (lacking ATG start codon), Hyg^r	This study
pSC301	pSODIT containing *gfp* from pGreenTIR (including ATG start codon, Shine Dalgarno, and TIR), Hyg^r	This study
pSC301b	Background fluorescence control for pSC301; pSC301 in which the SOD promoter was excised using *Xba*I and *Eco*RV, Vent polymerase blunt-ended, and re-ligated.	This study
pSC801	Transcriptional fusion to *gfp*; pSC301 containing the *M. tuberculosis* pknH gene upstream 636 bp inserted into the *Xba*I/*Eco*RV sites, Hyg^r	This study
pSC120	Translational fusion to *gfp*; pSC300 containing the first 95 codons of the *M. tuberculosis* choD gene inserted into the *Xba*I/*Eco*RV sites and modified to include the SOD promoter, Hyg^r	This study
Oligonucleotides		
SC1	5′-TCTAGAACCTGCACGACATCGCCTC-3′	This study
SC2	5′GATATCGTCCTGTGCGTCGCTCATC-3′	This study
SC3	5′-GATATCGCGGCACGTACAAC-3′	This study
SC4	5′-TCTAGAGCGGCCTGAATCTG-3′	This study

[a] Amp^r, ampicillin resistance; Hyg^r, hygromycin resistance; SOD, superoxide dismutase.
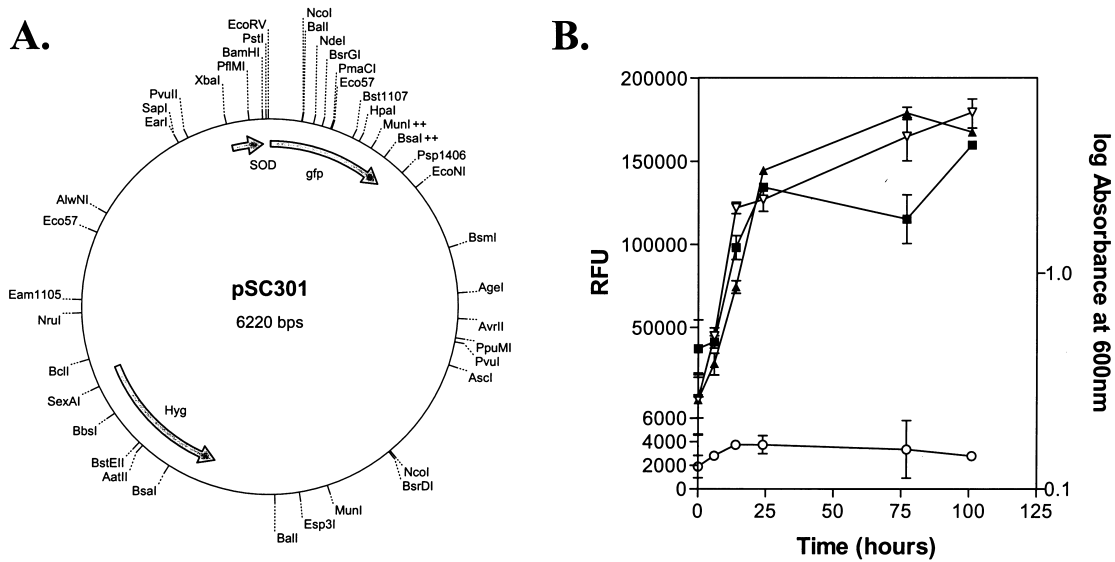
Fig. 1. Analysis of *gfp* expression by *M. tuberculosis* promoters using the transcriptional fusion vector pSC301 in *M. smegmatis*. (A) Restriction enzyme map of the *E. coli-Mycobacterium* transcriptional *gfp* fusion vector pSC301. Only unique enzyme sites are shown. (B) Spectrofluorometry of *M. smegmatis* cultures harboring pSC801 (*pkn*H promoter) or pSC301 (SOD promoter) during the growth cycle. RFU plotted on the left *y*-axis are shown for pSC801 (open circles) and pSC301 (closed squares), and OD at 600 nm are shown on the right *y*-axis for cultures of pSC801 (open triangles) and pSC301 (closed triangles). All measurements were carried out on triplicate cultures (± standard error) and were corrected for background fluorescence by subtracting the RFU for control strain pSC301b incubated under identical conditions. Abbreviations: Hyg, hygromycin resistance gene; SOD, superoxide dismutase promoter; *gfp*, green fluorescent protein gene; OD, optical density; RFU, relative fluorescence units.
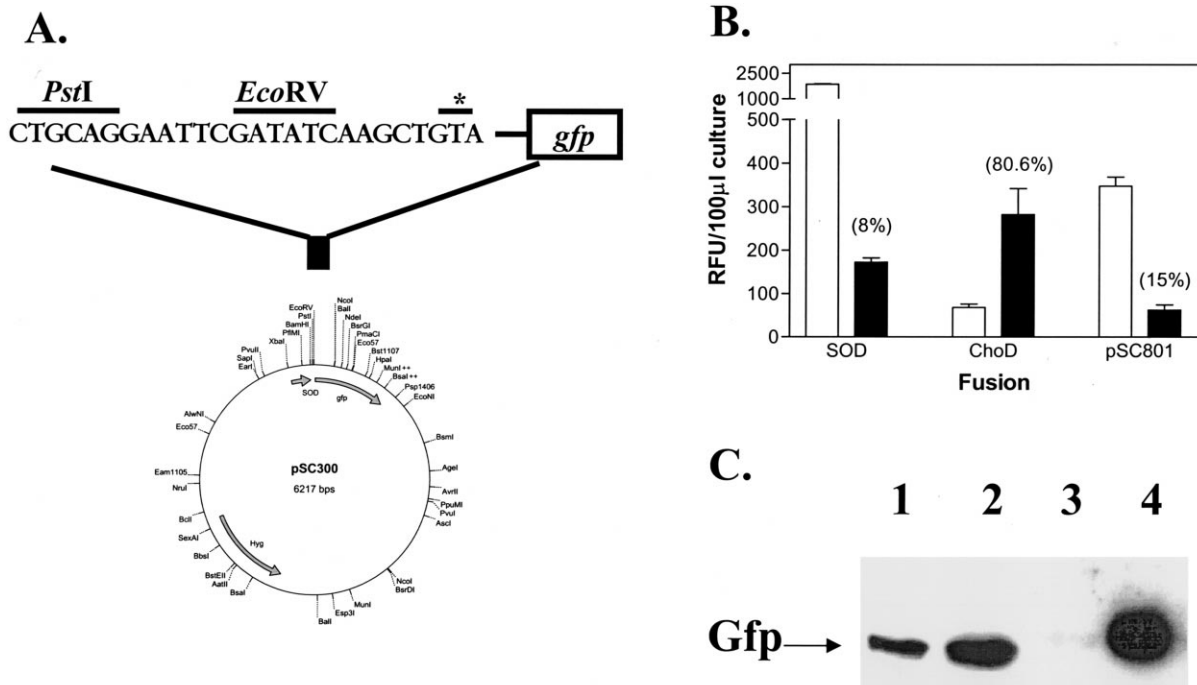

Fig. 2. Analysis of Gfp localization in *M. smegmatis* cultures harboring translational fusions to *gfp* in pSC300. (A). Restriction enzyme map of the *E. coli-Mycobacterium* translational *gfp* fusion vector pSC300. Only unique enzyme sites are shown. A magnification of the sequence of the region located near the beginning of the *gfp* gene (lacking an ATG codon) is provided. The location of the first codon of the *gfp* gene is indicated by a bar and an asterisk. (B). Spectrofluorometry of cells (white bars) and supernatant (black bars) from *M. smegmatis* cultures harboring transcriptional or translational *gfp* fusions as indicated. The percent of Gfp located in the supernatant for each culture is shown in parentheses. Data is representative of several experiments, and results are expressed as the averages of three determinations ± standard error. (C). Western immunoblot of a 7.5% SDS-PAGE gel using an anti-Gfp monoclonal antibody of fractionated cells (lanes 1 and 3) and supernatants (lanes 2 and 4) from *M. smegmatis* cultures harboring either pSC300 (Gfp; lanes 1 and 2), or pSC120 (ChoD-Gfp; lanes 3 and 4).

*pkn*H start codon and the sequences including the upstream 636 bp from the gene. This fragment was amplified from H37Rv genomic DNA using primers SC3 and SC4 (Table 1) designed to result in a 5′ *Xba*I site and a 3′ *Eco*RV site. The resulting PCR fragment was TA cloned into pCR2.1, and subsequently excised with *Xba*I and *Eco*RV, and ligated to *Xba*I and *Eco*RV cut pSC301 from which the SOD promoter had been excised to yield plasmid pSC801. The *ptpA-gfp* translational fusion was constructed by PCR amplification of a sequence from *M. tuberculosis* H37Rv genomic DNA that includes the *choD* start codon as well as the first 95 codons of coding sequence of the gene. This was done using primers SC1 and SC2 (Table 1) that had been designed to result in a 5′ *Bgl*II site and a 3′*Eco*RV site, and the PCR product was TA cloned into pCR2.1. To create the ChoD-Gfp fusion plasmid, pSC120, this PCR product was excised from pCR2.1 using *Bgl*II and *Eco*RV, and cloned into the corresponding *Bam*HI and *Eco*RV sites in pSC300. These constructs were electroporated into *M. smegmatis* or *M. bovis* BCG as previously described (Pavelka and Jacobs, 1996).

2.4. Gfp transcriptional fusion assays

Gfp fluorescence of bacterial cultures was quantified using a spectrofluorometer (BioRad VersaFluor™). Cells were grown as described, and at the indicated time intervals 100 µl of each culture was removed, diluted into 1–2 ml of PBS, and the relative fluorescence units (RFU) were measured using a 490/10 excitation filter and a 520/10 emission filter. The OD at 600 nm of each culture was measured for each culture in a Jenway 6405 UV/Vis. spectrophotometer. Background fluorescence due to read-through transcription of the transcriptional fusion vector was determined by measuring the RFU of strains subjected to the same conditions harboring the background control pSC301b plasmid (Table 1). Special conditions used to test promoter expression include exposure to microaerophilic conditions for 10 days as previously described (Cunningham and Spreadbury, 1998), heat shock (incubation at 42°C), oxidative stress (exposure to 10 mM H_2O_2), and acid stress (7H9 medium adjusted to a pH of 4.5). Cultures were exposed to the indicated conditions for a minimum of 4 h prior to removal of a 100 µl aliquot and measurement of RFU and OD at 600 nm.

2.5. Gfp translational fusion assays

The optical density at 600 nm was measured for triplicate cultures grown for 16 h 7H9 broth media, and the ODs were adjusted to 0.5 for each culture using additional 7H9. Cells from the OD-adjusted cultures were separated from the supernatant by centrifugation at $20,000 \times g$ for 15 min. The cells were then resuspended in the same volume of 7H9 broth media as the supernatant, and these cell and supernatant fractions were then subjected to either spectrofluorometry or Western immunoblot analysis. The fluores-

cence intensity of 100 µl of the separated cells and supernatant was determined by spectrofluorometry using a BioRad VersaFluor™ fluorometer. Western immunoblot analysis of the same translational fusion fractions was performed following loading of equal volumes of cell and supernatant fractions on a 7.5% SDS-PAGE gel. Samples were transferred to nitrocellulose using a semi-dry Western transfer apparatus, and blocked overnight at room temperature in Tris-buffered saline and 0.1% Tween-20 (TBS-T) with 10% skim milk. The blot was washed three times in TBS-T, then incubated for 4 h in TBS-T with a 1 in 1000 dilution of anti-Gfp monoclonal antibody (Boehringer Mannheim), washed again three times in PBS-T, then incubated for 1 h with a 1 in 1000 dilution of goat anti-mouse IgG antibody conjugated to horse radish peroxidase. Detection was performed using the Pierce chemiluminescent system according to the manufacturer's instructions.

2.6. THP-1 infections and confocal microscopy

The THP-1 cells were grown in RPMI 1640 containing 5% FBS and 0.05 mM β-mercaptoethanol. In a 24-well tissue culture plate, 10^5 cells were seeded onto glass coverslips in RPMI 1640 containing 5% FBS and 0.05 mM β-mercaptoethanol, then differentiated using 20 ng/ml phorbol myristate overnight. Infections were performed as previously described (Via et al., 1998a,b). After 24 h, the infected macrophages were fixed with 4% formaldehyde and visualized by confocal fluorescence microscopy. The bacteria examined were confirmed to be intracellular based upon their location within the slices of the sample.

3. Results and discussion

3.1. Construction of an E. coli-Mycobacterium Gfp fusion vector for monitoring promoter activity

Due to the low expression levels from many of the promoters in Mycobacterium species (Das Gupta et al., 1993), we reasoned that gene expression in mycobacteria would be easier to detect using a *gfp* gene that has been optimized specifically for use with bacterial promoters that confer low levels of transcription. Therefore, the enhanced *gfp* gene used here contains the S65T 'red shift' and F64L 'protein solubility' mutations that have been previously shown to exhibit 40–80-fold greater fluorescence than wild type *gfp* (Miller and Lindow, 1997). For the construction of the transcriptional fusions, the enhanced *gfp* gene from pGreenTIR was inserted into the *Hind*III site of the *E. coli*-Mycobacterium shuttle vector pSODIT (De Smet et al., 1999) (Table 1 describes the bacterial strains, oligonucleotides, and plasmids used in this study). This Gfp cassette includes an ATG translational start codon and a consensus Shine-Dalgarno with a spacer region sequence that minimizes RNA secondary structure. The resulting plasmid, named pSC301, thus contains an enhanced *gfp* gene located

downstream of a multiple cloning site and the *M. tuberculosis* superoxide dismutase (SOD) promoter (see Fig. 1A). The SOD promoter may be easily excised with a variety of enzymes and replaced with a mycobacterial promoter region of choice.

3.2. Transcriptional GFP fusions are expressed in M. smegmatis

In order to confirm that the transcriptional fusion vector pSC301 is functional, we replaced the SOD promoter in pSC301 with the upstream region from the *M. tuberculosis* gene for the serine/threonine protein kinase *pkn*H (Av-Gay and Everett, 2000) to yield plasmid pSC801. Plasmids pSC301 and pSC801 were then electroporated into *M. smegmatis*.

Epifluorescence microscopy of *M. smegmatis* harboring the transcriptional fusion vectors pSC301 and pSC801 revealed green fluorescence typical of GFP-producing bacteria. We used spectrofluorometry to measure *gfp* expression and compare the transcriptional activities of the genes for the *M. tuberculosis* protein kinase *pkn*H (pSC801) and the SOD promoter (pSC301) following exposure to a variety of stress conditions. The fluorescence levels of these promoters were first compared for *M. smegmatis* grown over a 100-hour period in bacteriological media. Fig. 1B indicates that *gfp* expression from the SOD promoter is extremely high as compared to the levels expressed from the *pkn*H promoter. *Mycobacterium smegmatis* cultures harboring pSC301 or pSC801 were then exposed to conditions of heat shock, low pH, oxidative stress, and microaerophilic conditions. As shown in Table 2, incubation at 42°C and exposure to low pH decreased transcription levels from both

Table 2
Promoter activity of *M. smegmatis* harboring *M. tuberculosis* promoter - *gfp* transcriptional fusions in response to stress conditions

Stress Condition[a]	pSC801 (*pkn*H – *gfp*)[b]	pSC301 (*SOD* – *gfp*)
Control (medium at 37°C)	100	100
Acid (medium adjusted to pH 4.5)	53.9	83
Heat (medium incubated at 42°C)	65	71
H_2O_2 (medium adjusted to 10 mM)	96.7	92
Microaerophilic growth – control	100	100
Microaerophilic growth – test	82	111

[a] Strains were exposed to the indicated conditions for a 4 h incubation period. Microaerophilic growth is reported for cultures grown to an OD at 600 nm of 0.5 and inoculated into 5 ml media in a 13 ml Sarstedt tube and incubated for 2 weeks at 37°C with shaking (aerobic control) or stationary (test).

[b] Values are reported as % promoter activity relative to the control culture, which was assigned a value of 100%. Promoter activity was measured as relative fluorescence units (RFU) and divided by the OD at 600 nm of the culture. All measurements were carried out on triplicate cultures and were corrected for background fluorescence by subtracting the RFU/OD600 nm for control strain pSC301b incubated under identical conditions.

the SOD and *pkn*H promoters to a significant extent. Conversely, exposure to 10 mM hydrogen peroxide had little effect on either promoter. The observed lack of up-regulation of the SOD promoter, a promoter known to be sensitive to oxidative stress (Harth and Horowitz, 1999), in response to the hydrogen peroxide used in this experiment is likely due to the presence of Gfp itself. The auto catalytic formation of the Gfp fluorophore produces hydrogen peroxide as a by-product in quantities that can sometimes be lethal to the bacterial host (Tsien, 1998; Miller and Lindow, 1997), and could be responsible for constitutive maximal SOD promoter activity. From these experiments we conclude that pSC301 is a functional *gfp* transcriptional fusion vector capable of distinguishing between a broad range of promoter activity levels in *M. smegmatis*. Furthermore, we conclude that the SOD promoter directs very high levels of transcriptional activity in *M. smegmatis* under a variety of stress conditions.

3.3. Translational Gfp fusions to M. tuberculosis proteins can be used to determine protein secretion in M. smegmatis

In order to construct a translational fusion vector, the *gfp* gene was excised from the plasmid pGreen and cloned into the blunt-ended pSODIT HindIII site. The resulting plasmid was named pSC300, and contains an enhanced *gfp* gene lacking translation initiation signals and an ATG start codon (Fig. 2a). This cassette is located directly downstream from the multiple cloning site as well as from the SOD promoter, thus allowing for efficient cloning of in-frame upstream mycobacterial genes. Expression of the resulting Mycobacterium-Gfp fusion protein may be driven by the strong SOD promoter present in pSC300, or, if desired, the SOD promoter may be removed and transcription of the fusion protein gene may be driven by the promoter associated with the gene of interest. In the absence of any gene cloned into pSC300, a short Gfp fusion protein is expressed due to the presence of an in-frame GTG codon located 24 nucleotides upstream of the gfp gene.

To demonstrate that Gfp can be used as a marker to study subcellular localization of *Mycobacterium* proteins, we constructed a translational fusion of Gfp to a predicted secreted protein in pSC300. As our candidate gene, we chose the *M. tuberculosis* cholesterol oxidase protein (ChoD) because the amino acid sequence for this protein contains a putative secretion signal sequence at its N-terminus, and it is expected to be secreted due to previous observations of ChoD activity in the extracellular medium of *M. tuberculosis* cultures (Av-Gay and Sobouti, 2000). Due to the presence of an N-terminal signal sequence, we reasoned that cloning the first 95 amino acids for this protein would be sufficient to direct secretion. Thus, we cloned the first 95 codons of *cho*D downstream from the high activity SOD promoter present in pSC300 to create the plasmid named pSC120. Plasmids pSC300 and pSC120 were then electroporated into *M. smegmatis*.

We separated cells and supernatant of *M. smegmatis* cultures harboring the Gfp fusion proteins. The resulting fractions were subjected to spectrofluorometry to quantify the levels of Gfp found in the cells versus the culture supernatant. As seen in Fig. 2b, only 8–15% of the Gfp was detected within the supernatant of cultures expressing the negative secretion control pSC300, as well as the transcriptional fusion pSC801. Cell lysis due to the toxic nature of overexpressed Gfp from the SOD promoter may have been responsible for the small amount of extracellular Gfp detected in these cultures. In contrast, 80% of the Gfp was detected in the supernatant of cultures expressing the ChoD-Gfp fusion protein. To confirm these results, we performed a Western immunoblot on fractionated cultures using an anti-Gfp monoclonal antibody. As seen in Fig. 2c, Gfp is detected entirely in the supernatant of cultures expressing ChoD-Gfp. Surprisingly, pSC300 cultures exhibit approximately equal amounts of Gfp in the cells and the culture supernatant, although 92% of the Gfp was detected within the same cells by spectrofluorometry. We hypothesize that this observed difference is due to proteolytic degradation of the intracellular Gfp, resulting in retention of fluorescence but loss of the monoclonal epitope. In support of this hypothesis, work done in our laboratory and others (D.B. Young, personal communication), have detected multiple bands on similar Western blots of *M. smegmatis* cultures expressing other Gfp fusion proteins (data not shown). Despite this possible complicating factor, the putative secreted ChoD-Gfp fusion is detected primarily outside the cells by both Western analyses and spectrofluorometry, and our negative secretion controls are detected primarily inside the cells by spectrofluorometry. From these results, we conclude that it is possible to detect secreted Mycobacterial proteins using a Gfp translational fusion.
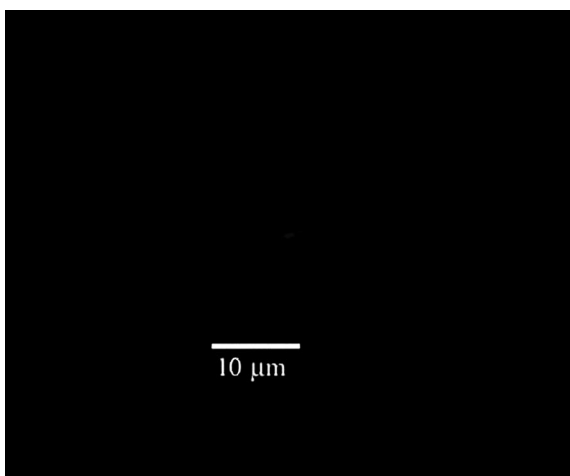

Fig. 3. Intracellular localization of *M. bovis* BCG harboring pSC300 during infection of PMA-differentiated THP-1 macrophages. Infected cells and multiplying bacteria were visualized at 24 h post-infection by confocal laser scanning fluorescence microscopy (MRC-600; Bio Rad Laboratories; Hercules, CA).

Ultimately, we wish to use this technology as a tool to follow protein expression and subcellular localization of Mycobacterial-Gfp fusion proteins within the host macrophage. As a potential practical application, Gfp fusion proteins secreted by *M. bovis* BCG or *M. tuberculosis* within the macrophage may be co-localized with other cellular markers using confocal fluorescence microscopy. Unfortunately, at this time we are unaware of any mycobacterial proteins that are targeted outside of the *Mycobacterium*-containing phagosome. Thus, in order to confirm that Gfp translational fusion proteins are visible within *M. bovis* BCG-infected macrophages, we electroporated pSC300 into *M. bovis* BCG and infected THP-1 cells. We then used confocal microscopy to examine the intracellular bacteria following 24 h of infection. Fig. 3 shows that the fluorescent bacilli are easily visible within the THP-1 cells, thus confirming that the use of translational Gfp fusion proteins may be a viable method to study intracellular targeting of mycobacterial proteins in the macrophage.

4. Conclusions

1. Here we have created a dual set of vectors for the creation of transcriptional (pSC301) and translational (pSC300) enhanced Gfp fusions for expression in *E. coli* and mycobacterial species. These two vectors can be used as a combined tool for fast analysis of promoter activity and protein localization within mycobacteria.
2. The transcriptional fusion vector pSC301 may be used to drive high levels of Gfp expression in *Mycobacterium* species from the SOD promoter, or, the SOD promoter may be excised and replaced for examination of levels of transcriptional activity from other mycobacterial promoters.
3. The translational fusion vector pSC300 may be used to create protein fusions to Gfp for expression in *E. coli* and *Mycobacterium* species. The fusion protein may be over expressed using the SOD promoter, or expressed using the endogenous promoter associated with the protein of interest in order to study its transcriptional and translational regulation. Expressed Gfp fusion proteins may be detected and localized using either monoclonal antibodies to Gfp, spectrofluorometry, or confocal fluorescence microscopy within infected cells.

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