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Highly fluorescent GFP_m^{2+}-based genome integration-proficient promoter probe vector to study *Mycobacterium tuberculosis* promoters in infected macrophages

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Summary

Study of activity of cloned promoters in slow-growing *Mycobacterium tuberculosis* during long-term growth conditions *in vitro* or inside macrophages, requires a genome-integration proficient promoter probe vector, which can be stably maintained even without antibiotics, carrying a substrate-independent, easily scorable and highly sensitive reporter gene. In order to meet this requirement, we constructed pAKMN2, which contains mycobacterial codon-optimized gfp_m^{2+} gene, coding for GFP_m^{2+} of highest fluorescence reported till date, mycobacteriophage L5 *attP-int* sequence for genome integration, and a multiple cloning site. pAKMN2 showed stable integration and expression of GFP_m^{2+} from *M. tuberculosis* and *M. smegmatis* genome. Expression of GFP_m^{2+}, driven by the cloned minimal promoters of *M. tuberculosis* cell division gene, *ftsZ* (*MtftsZ*), could be detected in the *M. tuberculosis*/pAKMN2-promoter integrants, growing at exponential phase in defined medium *in vitro* and inside macrophages. Stable expression from genome-integrated format even without antibiotic, and high sensitivity of detection by flow cytometry and fluorescence imaging, in spite of single copy integration, make pAKMN2 useful for the study of cloned promoters of any mycobacterial species under long-term *in vitro* growth or stress conditions, or inside macrophages.

Introduction

Episomal promoter probe vectors that are stably maintained with antibiotic selection are useful experimental systems to study promoter activity during short durations. However, to study activity of cloned promoters of a slow-growing pathogen, such as *Mycobacterium tuberculosis*, on a long-term basis *in vitro* or *in vivo* inside macrophages, it is difficult to maintain stable concentration of antibiotics. Such experimental systems need genome-integration proficient promoter probe vectors that can be stably maintained even without antibiotics for long durations. Vectors that integrate into mycobacterial genome through site-specific recombination via *attP* of mycobacteriophage L5 or Ms6 and *attB* in the presence of phage integrase, *int*, have been reported (Lee *et al*., 1991; DasGupta *et al*., 1998; Freitas-Vieira *et al*., 1998; Vultos *et al*., 2006). As a reporter, GFP and GFP mutants of higher fluorescence used for mycobacterial studies (Dhandayuthapani *et al*., 1995; Kremer *et al*., 1995; Luo *et al*., 1996; Valdivia *et al*., 1996; Parker and Bermudez, 1997; Barker *et al*., 1998; Via *et al*., 1998; Teitelbaum *et al*., 1999; Cowley and Av-Gay, 2001) offer the advantages of a sensitive, non-invasive, substrate-independent rapid assay system over β-galactosidase (Rowland *et al*., 1999), catechol-2,3-dioxygenase (Curcic *et al*., 1994), chloramphenicol acetyl transferase (DasGupta *et al*., 1993), and bacterial luciferase (Roberts *et al*., 2005). However, being single copy in the genome, a highly fluorescent GFP variant is required for the accurate determination of activity of promoters of different strength. In order to meet these requirements, the present study describes construction and use of pAKMN2, which combines the sensitivity of gfp_m^{2+} of highest fluorescence reported till date (Steinhauer *et al*., 2010) and L5*attP-int*-mediated stable genome-integration proficiency, to determine activity of cloned promoters of *M. tuberculosis* in *M. tuberculosis* on a long-term basis under *in vitro* growth conditions or inside macrophages even without antibiotics.

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Results

Construction and stability of, and expression from, genome-integrated pAKMN2

The construction of pAKMN2, from the source vectors, pMN406 (Roy *et al*., 2004) and pDK20 (DasGupta *et al*., 1998), through the intermediate episomal pAKMN1, is given in the self-explanatory Fig. 1. In order to verify the stability of genome-integrated pAKMN2-promoter constructs, *M. tuberculosis*/pAKMN2-P_{Q1K1} (Mt) and *M. smegmatis*/pAKMN2-P_{Q1K1} (Ms) integrants, carrying total promoter region, Q1-K1, of *M. tuberculosis* cell division gene, *ftsZ*, *MtftsZ* (Fig. 2A; Roy and Ajitkumar, 2005), were grown to mid-log phase without hygromycin and plated on hygromycin-containing and hygromycin-free plates. The colony-forming units (cfu) for both the integrants were comparable in the presence and absence of hygromycin (checked up to 30 and 60 generations for Mt and Ms integrants respectively) (Fig. 2B), with statistically insignificant values (two-sided *P*-values obtained by unpaired *t*-test: 0.3078 for Mt and 0.1374 for Ms). On the contrary, statistically significant reduction (two-sided *P*-values: 0.0008 for Mt and 0.0035 for Ms) in cfu was found in the absence of hygromycin for the episomal pMN406-P_{Q1K1} transformants of *M. tuberculosis* and *M. smegmatis*, grown under same conditions (Fig. 2B).

Flow cytometry of GFP_m^{2+} fluorescence in pAKMN2-P_{Q1K1} integrants of *M. tuberculosis* and *M. smegmatis* did not show significant difference in the presence or absence of hygromycin (Fig. 2B and C). However, there was significant reduction in the level of GFP_m^{2+} fluorescence in the absence of hygromycin for the episomal pMN406-P_{Q1K1}, in both *M. smegmatis* ($P = 0.0003$) and *M. tuberculosis* ($P = 0.0021$) (Fig. 2B and C). Comparison of the coefficient of variation (CV) of GFP_m^{2+} fluorescence intensities from Mt and Ms integrants, in the presence and absence of hygromycin, showed little difference (4% and 6% for Mt respectively; 5% and 7% for Ms respectively). Whereas, the CV of GFP_m^{2+} fluorescence intensities of episomal pMN406-P_{Q1K1} transformants of *M. tuberculosis* and *M. smegmatis* showed about two- to threefold increase (11% for Mt and 9% for Ms), in the presence of hygromycin. The CV values were even higher in the absence of hygromycin (46% for Mtb and 32% for Ms).

Promoter activity from M. tuberculosis*/pAKMN2 integrants* in vitro

Activity of individual cloned minimal *MtftsZ* promoters, P1, P2, P3, P4, P6, and total promoter, P_{Q1K1} (Roy and Ajitkumar, 2005), was determined for the respective *M. tuberculosis*/pAKMN2-promoter integrants using flow cytometry and semi-quantitative RT-PCR. P5 was not considered in the present study, as it was found to be active only in *M. smegmatis* but not in *M. tuberculosis* (Roy and Ajitkumar, 2005). Flow cytometry analyses of mid-log phase *M. tuberculosis*/pAKMN2-P1, -P2, -P3, -P4, -P6 and -P_{Q1K1} (positive control) integrants showed expression of GFP_m^{2+} (Fig. 2D). The vector control, *M. tuberculosis*/pAKMN2 (devoid of promoter), did not show GFP_m^{2+} expression (Fig. 2D). In order to verify the validity of the GFP based quantification of promoter activity, levels of gfp_m^{2+} mRNA in these integrants were determined using semi-quantitative RT-PCR, at the mid-linear range of amplification, taking P_{Q1K1} total promoter activity as 100% and *sigA* gene expression for normalization, as reported (Manganelli *et al*., 2001; Dubnau *et al*., 2002; Roy *et al*., 2004). Calculation of the % activities of P1, P2, P3, P4 and P6, obtained by flow cytometry and RT-PCR and comparison of the mean values themselves by one-way between-subjects ANOVA, followed by Post hoc comparisons using Tukey HSD test showed that RT-PCR and flow cytometry data were mostly consistent with each other (Fig. 2E). P1 and P3 were the most significantly less active than P2, P4 and P6, whereas the latter three were active more or less at the same level as detected by RT-PCR and flow cytometry (Fig. 2E). Only P1 and P3 were found to be significantly different by RT-PCR (red line), but not by flow-cytometry (not shown) due to large standard deviations in P1 flow cytometry values. Flow cytometry based quantification in the steady-state condition in general reflected RT-PCR quantification of steady-state RNA levels. However, coefficients of variation (CV) of flow cytometry for % activity of the promoters were higher than the same in RT-PCR.

Promoter activity from M. tuberculosis*/pAKMN2 integrants in macrophages*

Mycobacterium tuberculosis $H_{37}Ra$, which was used for the identification of *MtftsZ* promoters, infects macrophages (Falcone and Collins, 1997; Zhang *et al*., 2005). Therefore, the activity profile of the *M. tuberculosis* $H_{37}Ra$/pAKMN2-P1, -P2, -P3, -P4 and -P6 integrants was examined in RAW 264.7 (ATCC TIB71) macrophages, in comparison with those under exponential phase of growth. *Mycobacterium tuberculosis*/pAKMN2-P_{Q1K1} (total promoter region) and *M. tuberculosis*/pAKMN2 (without promoter) integrants were the positive and negative controls respectively. At 36 h post infection, fluorescence microscopy of bacteria inside macrophage and flow cytometry analyses of macrophage-derived *M. tuberculosis* promoter integrants showed expression from all the individual promoters and P_{Q1K1} (Fig. 3A). Calculation of the % activity from flow cytometry data and pairwise comparison of mean of % activity (as in Fig. 2E) for each promoter between infection and exponential growth phase showed significantly low

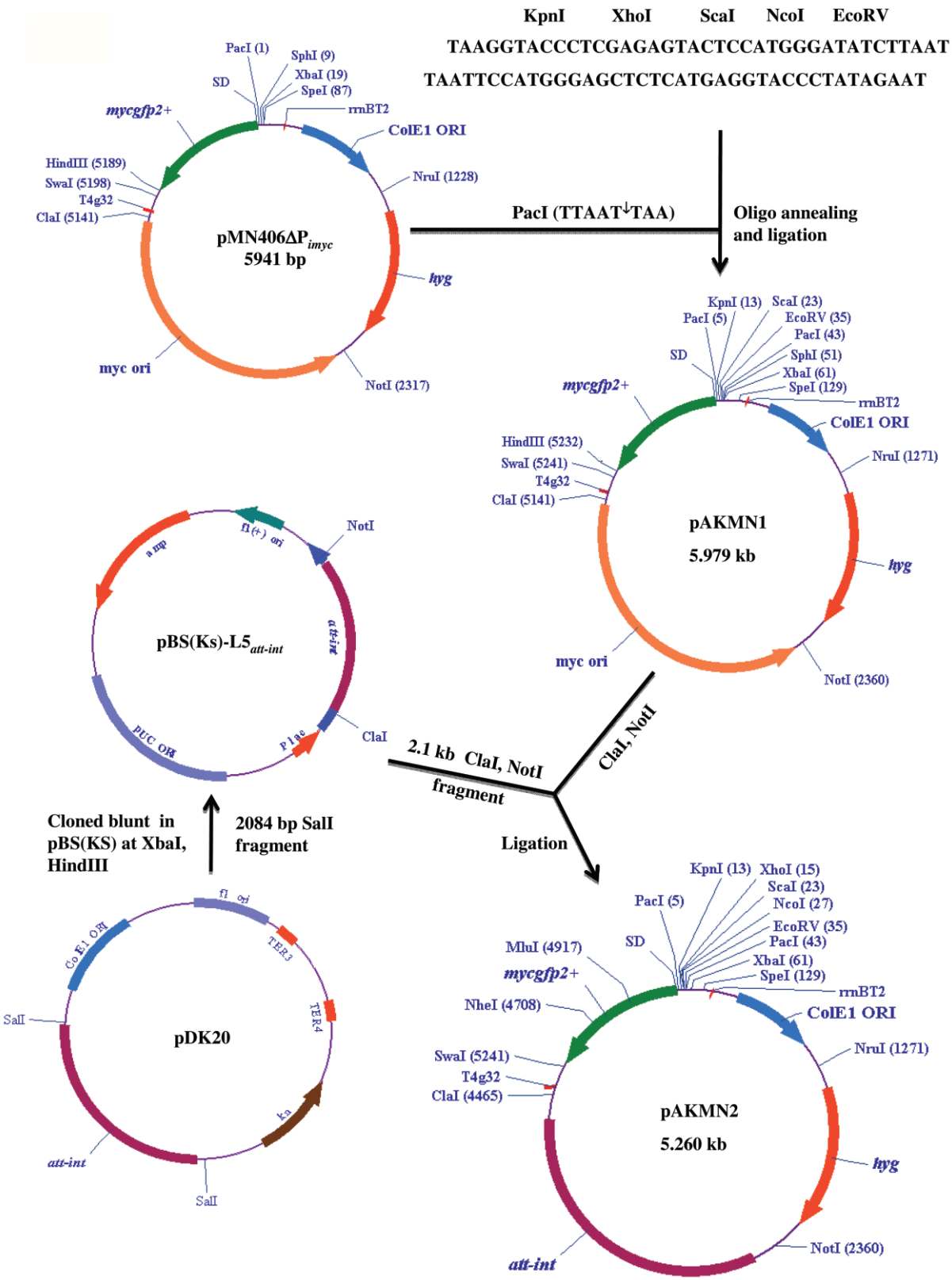
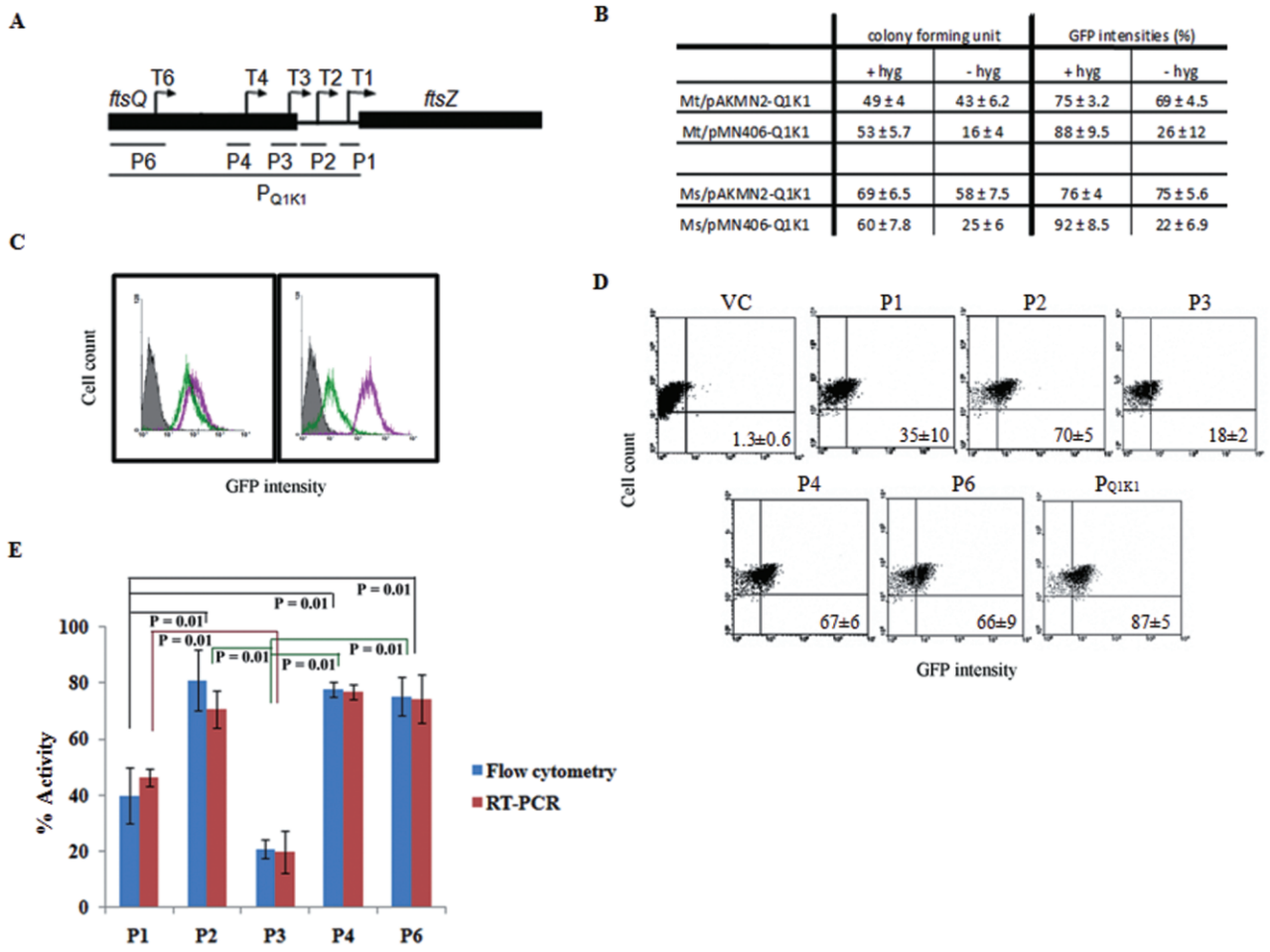


Fig. 1. Construction of pAKMN2 from the source vectors, pMN406-ΔP_{imyc} and pDK20, through the generation of the intermediate episomal vector, pAKMN1.



	colony forming unit		GFP intensities (%)	
	+ hyg	- hyg	+ hyg	- hyg
Mt/pAKMN2-Q1K1	49 ± 4	43 ± 6.2	75 ± 3.2	69 ± 4.5
Mt/pMN406-Q1K1	53 ± 5.7	16 ± 4	88 ± 9.5	26 ± 12
Ms/pAKMN2-Q1K1	69 ± 6.5	58 ± 7.5	76 ± 4	75 ± 5.6
Ms/pMN406-Q1K1	60 ± 7.8	25 ± 6	92 ± 8.5	22 ± 6.9

Fig. 2. A. Map of the transcription start sites (TSS) of T1–T4 and T6 and the span of their respective promoters, P1–P4, P6 and P_{Q1K1}.
B. First main column: Comparison of stability of pAKMN2-P_{Q1K1} integrant vector over episomal pMN406-P_{Q1K1} in the presence (+) or absence (–) of hygromycin (hyg) selection, in terms of cfu ($n = 3$). Second main column: GFP_m^{2+} intensities were calculated from flow cytometry results ($n = 3$) in both *M. smegmatis* (Ms) and *M. tuberculosis* (Mt) integrants of pAKMN2-P_{Q1K1}, in comparison to the respective transformants of episomal pMN406-P_{Q1K1} vector.
C. Representative histogram of a single data set of *M. tuberculosis*. Grey shaded histogram: exponential Mt; Green histogram:Mt/pAKMN2-P_{Q1K1} (left panel) or Mt/pMN406-P_{Q1K1} (right panel) in the absence of hygromycin; Purple: Mt/pAKMN2-P_{Q1K1} (left panel) or Mt/pMN406-P_{Q1K1} (right panel) in the presence of hygromycin.
D. Representative dotplots from single flow cytometric data set of P1–P4 and P6, vector control (pAKMN2, devoid of promoter), and *M. tuberculosis*/pAKMN2-P_{Q1K1} integrants. The mean and standard deviations for GFP intensities driven by the cloned promoters were shown as inset.
E. Comparison of % activity of P1-P4, and P6 obtained from flow cytometry (blue bar) and semi-quantitative RT-PCR (red bar) for gfp_m^{2+} in *M. tuberculosis*/pAKMN2-Promoter integrants in exponential phase. Only significant differences in activity between promoters were indicated by lines [black, green: significant difference obtained by both RT-PCR and flow cytometry for P1 (black) and P3 (green); red: differences obtained only by RT-PCR].

activity of P1 and P3 (two-sided *P*-value: 0.04 for P1 and 0.01 for P3; Fig. 3B). Statistically insignificant reduction was noticed in the % activity of P2, P4 and P6, compared with their activity under exponential phase. Similarly, % activity of the promoters were calculated from the semi-quantitative RT-PCR data for gfp_m^{2+} mRNA from the pAKMN2-promoter-integrants from macrophages and from exponential cultures and statistical significance was evaluated (Fig. 3C), as described (see Fig. 2E). The results showed that there were no significant changes in the expression of gfp_m^{2+} from P2, P4 and P6 promoter regions between exponential and infection phases. However, there was significant reduction in the activity of P1 (two-sided $P = 0.04$) and P3 (two-sided $P = 0.02$) in the infection phase. Again, flow cytometry data reflected promoter activity detected using RT-PCR on RNA from *M. tuberculosis*/pAKMN2-promoter integrants under infection phase.

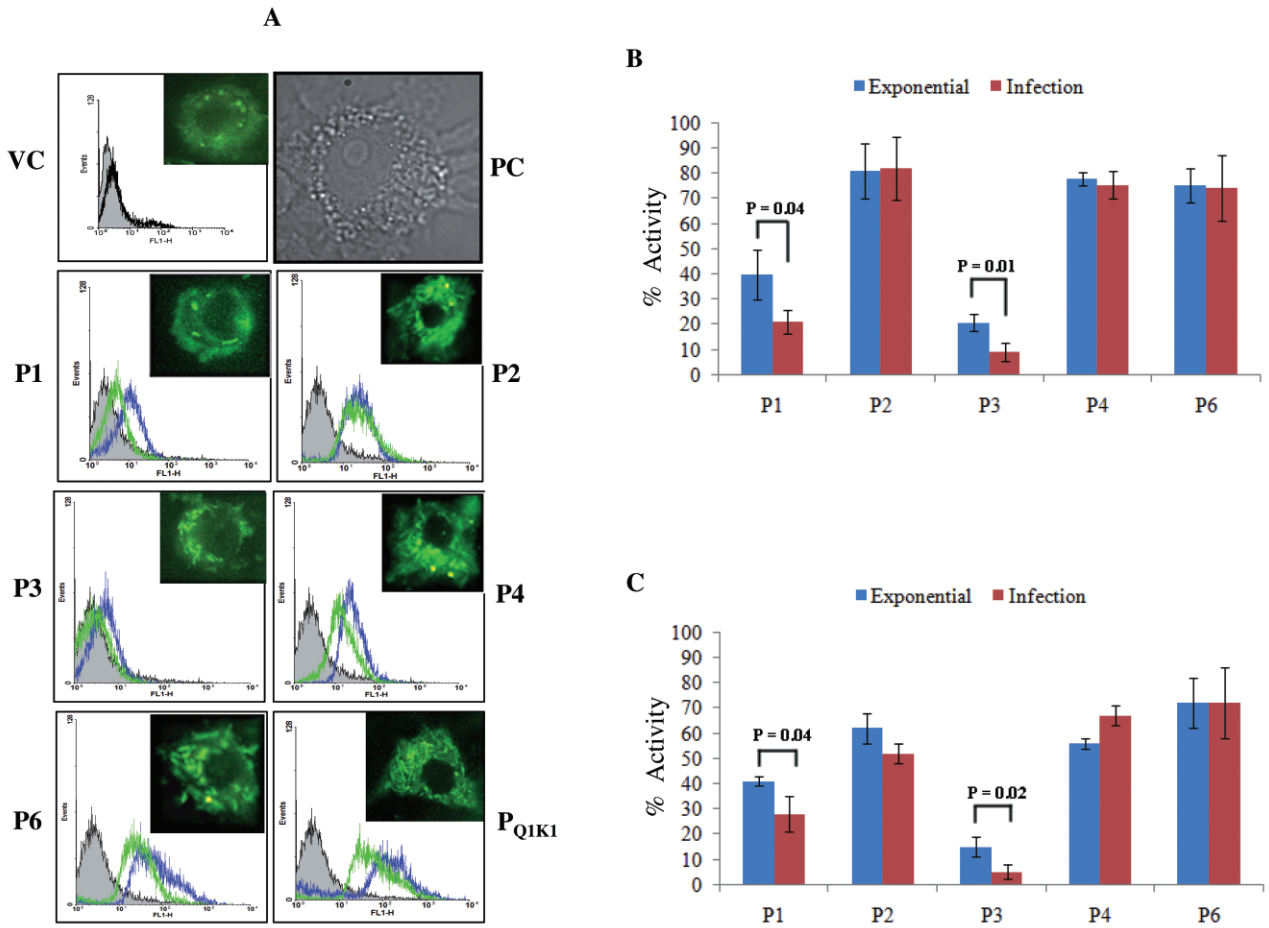


Fig. 3. A. Representative histograms from a single data set obtained by flow cytometry of GFP_m^{2+} expression from *M. tuberculosis*/pAKMN2-P1, -P2, -P3, -P4, -P6 and -P_{Q1K1} integrants in RAW 264.7 (ATCC TIB71) cells (green) and corresponding exponential cultures (blue) compared with each other and also to the pAKMN2 (vector control, VC). Panel VC: histogram (black line) of VC containing mycobacteria from infected macrophage, over exponentially grown untransformed wild-type mycobacteria (grey filled). P1–P4, P6 and P_{Q1K1}: VC, grey filled graph and green and blue lines as above. Fluorescence microscopy (inset): RAW 264.7 (ATCC TIB71) cells infected with *M. tuberculosis* pAKMN2-promoter integrants (100×). PC: phase contrast image of an infected macrophage cell.
B and C. Comparison of % activity of promoters based on flow cytometry (B) and semi-quantitative RT-PCR (C) analyses for gfp_m^{2+}, in *M. tuberculosis*/pAKMN2-promoter integrants in RAW 264.7 (ATCC TIB71) cells (red) and exponential *in vitro* culture of *M. tuberculosis*/pAKMN2-promoter integrants (blue).

Discussion

The data presented in the study demonstrate that pAKMN2 possesses all the features that are required for a promoter probe vector for the study of promoters of *M. tuberculosis* on a long-term basis *in vitro* or inside macrophages, even without antibiotic. First of all, pAKMN2 showed stable genome integration in mycobacteria and stable expression of gfp_m^{2+}, even in the absence of antibiotic. Second, being genome-integrated single copy, the requirement for a highly sensitive reporter gene to detect and quantify low-activity promoters, such as P1 and P3, could be achieved with the use of gfp_m^{2+}. Since GFP_m^{2+} has been demonstrated to be more fluorescent than the earlier higher fluorescent GFP_m^{+} and twice more fluorescent than E-GFP, and stable in expression in both slow- and fast-growing mycobacteria (Steinhauer *et al.*, 2010), its higher sensitivity of detection of *MtftsZ* promoters over other *gfp* variants or other conventional reporters was not addressed in this study. Third, in general, RT-PCR for gfp_m^{2+} expression from the cloned promoter regions correlated with those from flow cytometry analyses under steady-state conditions. However, noticeably, the CV values of flow cytometry for % activity of some of the promoters were higher than those determined using RT-PCR. Therefore, it may not be completely reliable to use GFP_m^{2+} for quantification of promoter activity in situations other than steady-state conditions, due to its stability. The data presented imply that pAKMN2 may be amenable for use in a multi-well plate format under

Table 1. Primers used in the study.

Name	Sequence	Use
revgfp2PE	5′-cggtgaacagctcctcgcccttcgac-3′	MCS, sequencing
Q1	5′-gcgggatccgatatcatgacggaacacaacgaggacccacagatcgagcgc-3′	Cloning P6
P1P2r	5′-gggtactgccgctgcacccgcgcac-3′	Cloning P6
mgfp2	5′-gcctctagacttgtacagctcgtccatgccgtgggtga-3′	PCR
mgfp1	5′-ggcgaattcggtaccatgtcgaagggcgaggagctgttcaccggc-3′	RT-PCR
sigA1	5′-gctgctgcaggacctgggccgcgag-3′	RT-PCR
sigA2	5′-cgccgtagacctggccgatctcgtc-3′	RT-PCR

fluorescence microscope and to screen for promoters of genes that are active in infection or dormancy or starvation or stationary phase. It also offers the possibility to transform pAKMN2-promoter integrants with a multicopy plasmid containing a candidate regulatory gene or a genomic/cDNA library under an inducible promoter, to find out or screen for regulatory proteins of the promoter that could be of therapeutic target value.

Experimental procedures

Bacteria, media and culture

Mycobacterium tuberculosis H_{37}Ra and *M. smegmatis* mc^2155 cells were grown in Middlebrook 7H9 (Difco) liquid medium supplemented with 0.2% glycerol and 0.05% Tween 80 or in Middlebrook 7H10 agar (Difco) medium. *Mycobacterium tuberculosis* cultures were supplemented with 10% albumin-dextrose-catalase (ADC) in liquid medium and 10% oleic acid-albumin-dextrose-catalase (OADC) in solid medium. Hygromycin was used at 50 μg ml^{-1} in mycobacteria.

Plasmid constructs and molecular cloning

pMN406-P1, -P2, -P3, -P4 and -P_{Q1K1}, carrying the individual *MtftsZ* promoters, P1, P2, P3, P4 and P_{Q1K1} (the total promoter region encompassing all the promoters) cloned in place of P_{imyc} promoter of pMN406, upstream of gfp_m^{2+}, were already available (Roy and Ajitkumar, 2005). pMN406 contains mycobacterial plasmid *ori*, *mycori*, and ColE1 *ori*, hyg^r marker, and mycobacterial codon usage adapted gfp_m^{2+} that codes for GFP_m^{2+} (Steinhauer *et al*., 2010). pMN406-ΔP_{imyc} is pMN406 devoid of P_{imyc} promoter (Roy *et al*., 2004). pMN406-P6 was constructed by the PCR amplification of P6 using primers Q1 and P1P2r (Table 1) to obtain a 545 bp PCR product, which was digested with EcoRV and BclI and the final 389 bp region was cloned between the SspI-BamHI sites in pMN406, in place of P*imyc* promoter. Since pMN406-P1, -P2, -P3, -P4, -P6 and -P_{Q1K1} were already available, instead of cloning the respective promoters into pAKMN2, the 2.084 kb L5 *attP-int* region was subcloned from pBS(KS)-L5*att-int* as ClaI-NotI fragment and inserted into these vectors, replacing the *mycori* region from the respective constructs, to generate pAKMN2-P1, -P2, -P3, -P4, -P6 and -P_{Q1K1}. The Shine–Dalgarno sequence of gfp_m^{2+}, downstream of the MCS, helps in the transcriptional fusion. Transcription termination sequence of T4g32 gene (terminator from gene *32* of phage T4) and transcription terminator *rrn*BT2 separate the cloned promoter-*gfp* fusion cassette from the rest of the vector backbone. Presence of the integrants in the genomic DNA was verified using PCR with vector-specific mgfp2 primer (Table 1) and forward primer specific for the respective promoter region (Roy and Ajitkumar, 2005).

Macrophage cell infection with M. tuberculosis

The infection of mouse macrophage cell line RAW 264.7 (ATCC TIB71) with *M. tuberculosis* H_{37}Ra cells was carried out, as described (Butcher *et al*., 1998). In brief, RAW 264.7 cells were grown to semi-confluence in either 175 cm^2 flasks (for RNA isolation) or 24-well tissue culture plates containing #1 thickness 12 mm diameter glass cover glass (for microscopy) or 75 cm^2 flasks (for flow cytometry) in complete RPMI-1640 medium, containing 10% foetal calf serum. *Mycobacterium tuberculosis* promoter-integrants were washed three times in PBS to remove traces of antibiotic and sonicated for 5×5 s at 50% output to disperse clumps. Macrophage cells were infected with the bacterial cells at a multiplicity of infection of 10 and allowed to phagocytose the bacilli for 6 h in humidified 5% CO_2 incubator at 37°C. The cells were washed three times to remove un-phagocytosed bacteria and the medium was replaced with fresh RPMI-1640 medium containing 50 μg ml^{-1} gentamicin and incubated for a total period of 36 h post infection for analysis.

Preparation of cells for flow cytometry and fluorescence microscopy

Mycobacterium tuberculosis integrant cells were harvested at different stages of growth *in vitro* or from infected macrophages, washed once with PBS, finally resuspended in 1 ml PBS, for flow cytometry analysis. *Mycobacterium tuberculosis* cells from infected macrophage cells were harvested by osmolysing macrophage cells with sterile double-distilled water containing 0.1% Triton X-100. The bacterial cells were recovered by centrifugation at 8000 *g* for 15 min at 4°C, washed thrice with PBS, and resuspended in 500 ml PBS for flow cytometry and fluorescence microscopy. Flow cytometry was performed using Becton Dickinson FACScan machine. Dotplots were analysed using WinMDI software, version 2.8. GFP_m^{2+} fluorescence of the bacilli in infected macrophages were observed using Leica Microscope (DMLB) and images were captured.

RNA isolation and semi-quantitative RT-PCR

Total RNA isolation from *M. smegmatis* and *M. tuberculosis* cells was carried out, as described earlier (Roy *et al*., 2004) or using Tri-reagent (Sigma) according to manufacturer's instructions. Isolation of bacteria from macrophages for RNA extraction was carried out, as described (Dubnau *et al*., 2002). In brief, the macrophage cells were lysed by suspending in Tri-reagent. The lysate was centrifuged at 5000 *g* for 20 min to pellet down intracellular mycobacteria. RNA from the pelleted bacteria was extracted using freshly added Tri-reagent. RNA samples were treated with DNaseI (USB), in the presence of 1 unit of Procine (RNase inhibitor) (USB) per μg of RNA, to remove DNA contamination. Integrity of the RNA was verified on formaldehyde agarose gel and quantified. Semi-quantitative RT-PCR reactions were performed on 50–200 ng RNA samples, using Qiagen One-Step RT-PCR Kit, as described (Roy *et al*., 2004), with the primers mgfp1 and mgfp2 (Table 1). As normalization control, *sigA* was amplified, using sigA1 and sigA2 (Table 1). RT-PCR was carried out at the linear range of amplification and the bands on agarose gel were quantified (ImageQuant software, V 2.54).

Statistical analyses

The percentage activity of each promoter was calculated based on the total activity of Q1K1 promoter region in the same species in both flow cytometry and RT-PCR from minimum three sets of independent experiments. Activity of promoter obtained by flow cytometry or RT-PCR in a given species was compared with each other by one-way analyses of variance (ANOVA) with post-hoc adjustment for multiple comparisons after Tukey HSD test. For pairwise comparison unpaired *t*-tests were applied to obtain two-sided *P*-values.

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