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Variable Expression Patterns of *Mycobacterium tuberculosis* PE_PGRS Genes: Evidence that PE_PGRS16 and PE_PGRS26 Are Inversely Regulated In Vivo†

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Evaluation of expression of 16 PE_PGRS genes present in *Mycobacterium tuberculosis* under various growth conditions demonstrated constitutive expression of 7 genes, variable expression of 7 genes, and no expression of 2 genes. An inverse expression profile for genes PE_PGRS16 and PE_PGRS26 was observed to occur in macrophages and in mice infected with *M. tuberculosis*. Variable expression of PE_PGRS proteins could have implications for their role in the immunopathogenesis of tuberculosis.

The PE_PGRS genes of *Mycobacterium tuberculosis* are a family of 63 genes found dispersed throughout the genome of *M. tuberculosis* (5, 11) and the genome of *M. bovis* (13). Other mycobacteria, such as *M. avium*, lack PE_PGRS genes, as evidenced by the lack of reactivity with a molecular probe constructed from the polymorphic GC-rich sequence (PGRS) found in all PE_PGRS genes (21). Evidence to date suggests that certain PE_PGRS proteins are found at the surface of mycobacteria (1, 7) and that they have some role in mediating interactions with eukaryotic cells (4). We have recently shown that expression of a PE_PGRS gene in an *M. smegmatis* strain, which does not normally express any PE_PGRS genes, enhances the persistence of the recombinant mycobacteria within macrophages and mouse tissues (8). Together with earlier evidence which showed that certain PE_PGRS genes are specifically expressed by *M. marinum* in granulomas (18), the current body of evidence indicates that differential expression of PE_PGRS proteins could have a role in the pathogenesis of tuberculosis and in altering the way the host responds to infection. In order to better understand the function and regulation of PE_PGRS proteins, we have investigated the expression of approximately one-third of the PE_PGRS genes present in *M. tuberculosis* cultured under different environmental conditions in vitro and following infection of primary macrophages or mice with the pathogen.

Reverse transcriptase PCR (RT-PCR) techniques were used to examine the expression of 16 PE_PGRS genes in *M. tuberculosis* strains CDC1551, Erdman, H37Rv, and HN878 as well as in *M. bovis* BCG Pasteur cultured in vitro under various growth conditions as described previously (8). Duplex RT-PCR was performed by including the 16S rRNA gene-specific internal primers to evaluate the relative expression levels of PE_PGRS genes. Primers were selected using the known gene sequences from *M. tuberculosis* strains H37Rv (5) and CDC 1551 (11) to specifically amplify fragments of individual PE_PGRS genes ranging between 300 and 800 bp in size (see Table S1A in the supplemental material). In all cases, the PCR products were sequenced to confirm the identity of the specific PE_PGRS gene and assays were performed at least three times on different biological samples. As shown in Fig. 1A, *M. tuberculosis* CDC1551 grown under log-phase culture conditions expressed 14 of the 16 PE_PGRS genes tested. PE_PGRS27 and PE_PGRS50 were not expressed in vitro. Similar results were obtained with the *M. tuberculosis* laboratory strains H37Rv and Erdman by employing a semiquantitative duplex RT-PCR method (data not shown). By use of duplex RT-PCR for the *M. tuberculosis* strains Erdman and HN878 and *M. bovis* BCG Pasteur, the PE_PGRS expression profiles were found to be similar (Fig. 1B) except that in addition to the lack of PE_PGRS27 and PE_PGRS50 expression, strain HN878 did not express PE_PGRS55, although PCR amplification of genomic DNA demonstrated that the PE_PGRS55 gene is present. In addition, since PE_PGRS35 is found in RD2, a genomic region missing in *M. bovis* BCG strains obtained after 1927 (2), there was no expression of PE_PGRS35 by *M. bovis* BCG Pasteur (Fig. 1B).

The expression of PE_PGRS genes was monitored when *M. tuberculosis* strains Erdman and CDC1551 were cultured under conditions that mimic intracellular stress, including conditions of low oxygen as described by Wayne and Hayes (25), nutrient starvation as defined by Betts et al. (3), and low pH (pH 4.5) (23). Growth of the mycobacteria under these conditions (as determined by CFU measurements) was inhibited after 15 days in culture, but viable bacteria were observed for up to 60 days in culture (data not shown). Samples were collected for RT-PCR at 5, 30, and 60 days, RNA was extracted, and duplex PCR was performed on quantitatively identical samples of RNA to compare levels of expression of the PE_PGRS genes relative to that of the 16S rRNA gene. Figure 2 shows typical results obtained by duplex RT-PCR comparing *M. tuberculosis*

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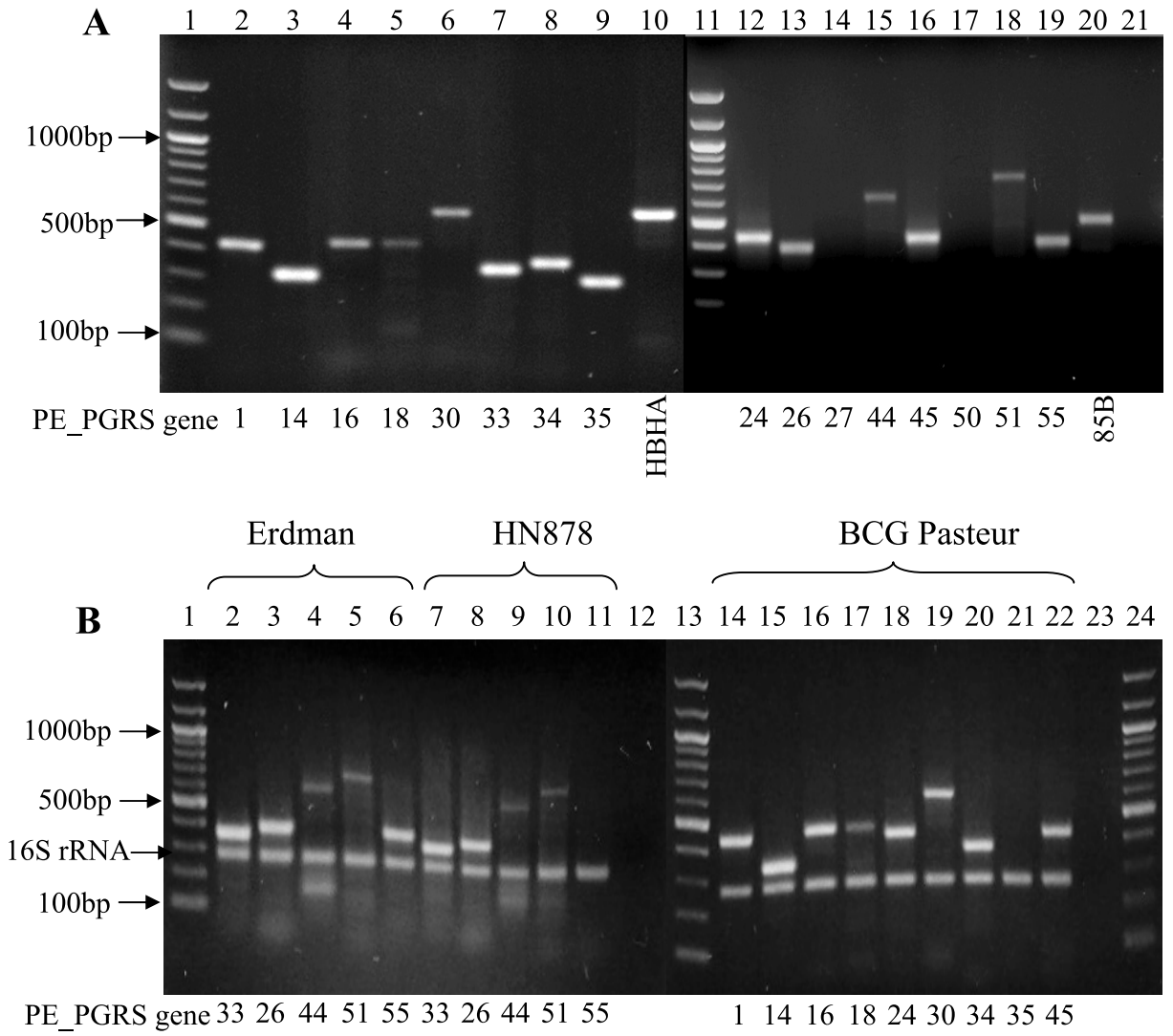

FIG. 1. Expression of PE_PGRS genes by mycobacteria in log-phase culture, as analyzed by RT-PCR. Specific primers were used to amplify internal fragments of PE_PGRS genes, and individual products were verified by sequencing. (A) Agarose gel showing the in vitro expression of PE_PGRS genes by *M. tuberculosis* CDC1551 growing in log-phase culture. Lanes 2 to 9 and 12 to 19 show the RT-PCR products of 16 PE_PGRS genes as indicated; lanes 10 and 20 show the expression of the unrelated mycobacterial antigens HBHA (600 bp) and 85B (491 bp) as RT-PCR controls; lane 21 is a negative control containing a primer pair but no cDNA; and lanes 1 and 11 contain a 100-bp DNA ladder, as indicated by the arrows. (B) Agarose gel showing expression of selected PE_PGRS genes by *M. tuberculosis* strains Erdman (lanes 2 to 6) and HN878 (lanes 7 to 11) and *M. bovis* BCG Pasteur (lanes 14 to 22) growing in log-phase culture and examined by duplex RT-PCR using the 16S rRNA gene for comparison. Lanes 12 and 23 are negative controls containing a primer pair but no cDNA, while lanes 1, 13, and 24 contain a 100-bp DNA ladder (arrows). The position of the 16S rRNA gene in each lane is indicated.

strain CDC1551 that has been cultured for 30 days under hypoxic or nutrient-starved conditions with the same strain cultured for 5 days. Expression of PE_PGRS44 and PE_PGRS51 was not detected with growth under any of the stress conditions at 5 or 30 days (Fig. 2A and B). PE_PGRS26 expression was reduced at day 30, and PE_PGRS55 was not expressed when *M. tuberculosis* was grown under conditions of oxygen depletion (Fig. 2A) and was significantly reduced when nutrients were limiting (Fig. 2B) or in low pH (data not shown). The same results were found with *M. tuberculosis* strain Erdman, and the results expressed semiquantitatively as a ratio of PE_PGRS gene expression to 16S rRNA gene expression are summarized in Table 1. In addition to the reduced expression or lack of expression of PE_PGRS26, PE_PGRS44, PE_PGRS51, and PE_PGRS55, the results show that there was increased expression of PE_PGRS16 under nutrient-depleted conditions and of PE_PGRS18 under low-pH conditions compared with expression under log-phase, nutrient-rich growth conditions.

To determine expression of the 16 PE_PGRS genes by *M. tuberculosis* residing within macrophages, primary bone marrow macrophages (BMMO) were isolated from C57BL/6 mice as described previously (6) and infected with *M. tuberculosis* Erdman at a multiplicity of infection of 2:1 for 2 h. Bacteria were collected from lysed BMMO at 1, 3, and 6 days, and extracts were prepared for RNA analysis. Eight of the 16 PE_PGRS genes, such as PE_PGRS33 (Fig. 3), showed no

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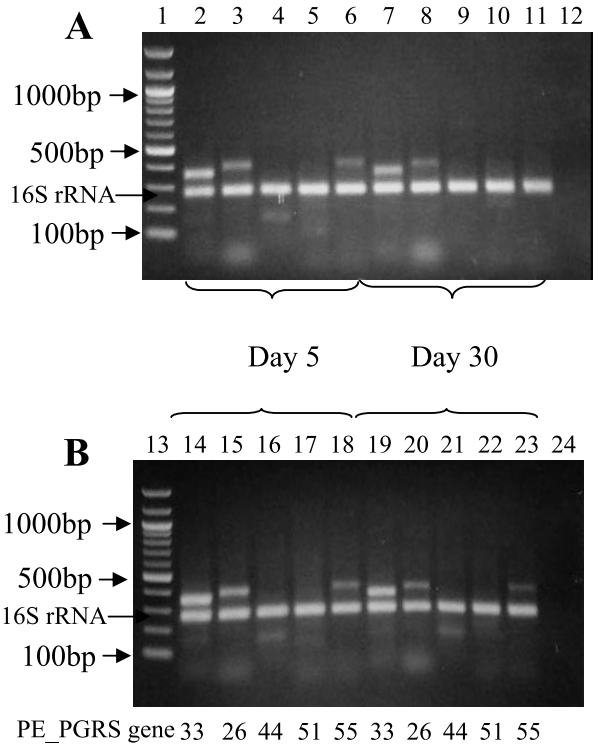

FIG. 2. Expression of selected PE_PGRS genes of *M. tuberculosis* strain CDC1551 cultured under conditions of hypoxia or nutrient depletion, as analyzed by duplex RT-PCR. Expression was determined for *M. tuberculosis* strains cultured under conditions of (A) hypoxia (lanes 2 to 11) or (B) nutrient depletion (lanes 14 to 23) after 5 and 30 days. Coexpression of the 16S rRNA gene (indicated at left) was used as a comparison to determine the relative expression levels of the PE_PGRS genes. Lanes 2, 7, 14, and 19, PE_PGRS33; lanes 3, 8, 15, and 20, PE_PGRS26; lanes 4, 9, 16, and 21, PE_PGRS44; lanes 5, 10, 17, and 22, PE_PGRS51; lanes 6, 11, 18, and 23, PE_PGRS55. Lanes 1 and 13 contain a 100-bp DNA ladder (arrows), and lanes 12 and 24 are a no-cDNA template control.

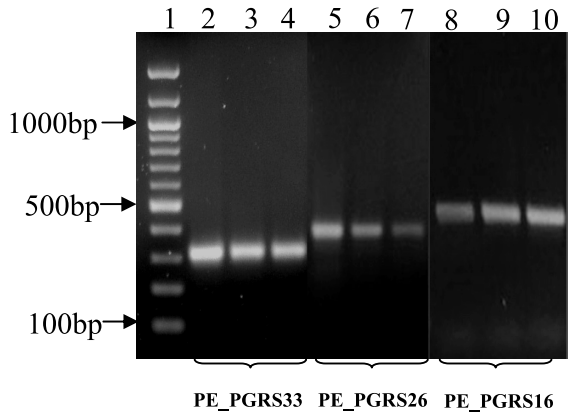

FIG. 3. Expression of PE_PGRS genes by *M. tuberculosis* strain Erdman residing within murine BMMO. RT-PCR-determined expression of genes PE_PGRS33, PE_PGRS26, and PE_PGRS16 in bone marrow macrophages infected with *M. tuberculosis* Erdman for 3 or 6 days compared with expression in mycobacteria growing in culture is shown. Lane 1, 100-bp DNA ladder (arrows); lanes 2, 5, and 8, *M. tuberculosis* Erdman in typical log-phase culture; lanes 3, 6, and 9, *M. tuberculosis* Erdman from BMMO after 3 days; lanes 4, 7, and 10, *M. tuberculosis* Erdman from BMMO after 6 days.

significant change in expression after residing within macrophages for 6 days. PE_PGRS44 and PE_PGRS51 showed no detectable expression after 6 days in macrophage cultures, while PE_PGRS1, PE_PGRS26, and PE_PGRS55 showed reduced expression in macrophages (Fig. 3; Table 1). For only one gene, PE_PGRS16, was there a suggestion of enhanced expression within macrophages, and there continued to be no detectable expression of PE_PGRS27 or PE_PGRS50 (Fig. 3; Table 1). The suspected induction of PE_PGRS16 expression and loss of expression of PE_PGRS26 by *M. tuberculosis* residing within macrophages was investigated using real-time RT-PCR on RNA samples from *M. tuberculosis* Erdman re-

TABLE 1. Expression of PE_PGRS genes by *M. tuberculosis* grown under various culture conditions, relative to 16S rRNA gene expression[a]

Gene name	Synonym	PE_PGRS gene expression under culture condition[b]				
		Log phase (6–10 days)	Hypoxia (30 days)	Nutrient depletion (30 days)	Low pH, 4.5 (30 days)	Intracellular growth in BMMO (6 days)
PE_PGRS01	Rv0109	E (1.82)	E (0.83)	E (1.24)	E (1.34)	LE (0.46)
PE_PGRS14	Rv0834c	HE (2.19)	E (1.10)	HE (2.56)	E (1.26)	E (1.30)
PE_PGRS16	Rv0977	E (1.10)	E (0.76)	HE (2.35)	E (1.14)	HE (2.72)
PE_PGRS18	Rv0980c	LE (0.26)	LE (0.15)	E (0.84)	E (1.23)	LE (0.26)
PE_PGRS24	Rv1325c	E (1.60)	E (0.75)	E (1.09)	E (1.76)	E (0.68)
PE_PGRS26	Rv1441c	E (1.77)	LE (0.46)	LE (0.29)	E (0.58)	LE (0.38)
PE_PGRS27	Rv1450c	NE (0)	NE (0)	NE (0)	NE (0)	NE (0)
PE_PGRS30	Rv1651c	E (0.99)	E (0.91)	E (0.73)	E (1.33)	E (0.74)
PE_PGRS33	Rv1818c	E (1.82)	E (0.73)	E (0.88)	E (1.04)	E (0.90)
PE_PGRS34	Rv1840c	HE (2.04)	E (1.17)	HE (2.11)	E (1.84)	E (1.23)
PE_PGRS35	Rv1983	HE (2.67)	E (1.25)	E (1.74)	E (1.92)	E (1.25)
PE_PGRS44	Rv2591	E (0.71)	NE (0)	NE (0)	NE (0)	NE (0)
PE_PGRS45	Rv2615c	E (0.98)	E (0.78)	E (1.30)	E (0.98)	E (1.12)
PE_PGRS50	Rv3345c	NE (0)	NE (0)	NE (0)	NE (0)	NE (0)
PE_PGRS51	Rv3367	E (0.52)	NE (0)	NE (0)	NE (0)	NE (0)
PE_PGRS55	Rv3511	E (1.51)	NE (0)	LE (0.14)	E (0.5)	LE (0.16)

[a] Shown are differential expression patterns of PE_PGRS genes of *M. tuberculosis* Erdman relative to expression of the 16S rRNA gene, as studied by duplex RT-PCR. Selected PE_PGRS genes of *M. tuberculosis* Erdman were studied under various physiological conditions. The expressed gel bands were semiquantitated as net intensity (pixel) by using Kodak 1D image analysis software.

[b] The PE_PGRS gene expression levels relative to the 16S rRNA gene expression level are shown in parentheses and are defined as follows: HE, high expression (>2.0); E, expression (0.5 to 2.0); LE, low expression (<0.5); NE, not expressed (0).

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covered from BMMO at various times following infection. Quantitative expression of the PE_PGRS and 16S rRNA genes was performed using an i-Cycler iQ system (Bio-Rad Laboratories, Hercules, CA) (22), and the PE_PGRS-specific primer pairs and TaqMan probes were designed with Beacon Designer 2 software (Premier Biosoft International, Palo Alto, CA) (see Table S2A in the supplemental material). The amplification efficiency for each gene was determined (16), and the relative mRNA expression levels of the PE_PGRS genes were calculated as described elsewhere (17). Figure 4A shows that there was a gradual and significant induction of expression of PE_PGRS16 (~8-fold by day 5) and, conversely, a gradual significant reduction in the expression of PE_PGRS26 (~4-fold by day 5) by *M. tuberculosis* Erdman residing within primary macrophages over a 240-h time period. In comparison, a significant reduction in expression of PE_PGRS44, PE_PGRS51, and PE_PGRS55 was observed soon after infection of macrophages.

To evaluate in vivo expression of genes PE_PGRS16 and PE_PGRS26 following infection, mice (five per group) were aerogenically infected with *M. tuberculosis* Erdman, and lungs and spleens were isolated at different time points and homogenized for the determination of bacterial loads and extraction of total RNA (15). Colonization ranged from 6.2 to 6.0 log CFU in the lung and 4.3 to 5.1 log CFU in the spleen over the time period of 14 to 150 days following infection. In vivo expression of PE_PGRS16 and PE_PGRS26 in spleen and lungs are provided as a ratio compared to the expression of the 16S rRNA gene by real-time RT-PCR (22) (Fig. 4B and C). Expression of PE_PGRS16 increased significantly (~5-fold) over the time period of 14 to 150 days in spleens of mice and slightly in lungs, and expression remained relatively high through 150 days of infection. Alternatively, expression of PE_PGRS26 decreased about threefold in spleens and about twofold in lung tissues over the 150-day time period.

The major findings of this study demonstrate (i) that 7 of the 16 PE_PGRS genes, PE_PGRS14, PE_PGRS24, PE_PGRS30, PE_PGRS33, PE_PGRS34, PE_PGRS35, and PE_PGRS45, were constitutively expressed under all of the in vitro growth conditions examined; (ii) that PE_PGRS44 and PE_PGRS51, which were reasonably expressed in log-phase, nutrient-rich culture, showed little or no expression under all other growth conditions examined; (iii) that PE_PGRS26 and PE_PGRS55 were significantly reduced under all of the in vitro conditions tested; (iv) that three genes, PE_PGRS1, PE_PGRS26, and PE_PGRS55, showed reduced expression and expression of one PE_PGRS gene, PE_PGRS16, was significantly induced by *M. tuberculosis* persisting within macrophages; and (v) that two genes, PE_PGRS27 and PE_PGRS50, were silent under all growth conditions tested. Of considerable interest was the finding that *M. tuberculosis* residing within macrophages increased the expression of PE_PGRS16 ~8-fold while concurrently decreasing the expression of PE_PGRS26 ~4-fold over a period of 5 days in culture. This inverse regulation of PE_PGRS16 and PE_PGRS26 was also observed for mouse tissues infected with *M. tuberculosis*.

In the studies presented here, a number of PE_PGRS genes were expressed by all strains of mycobacteria tested, under all of the in vitro conditions examined, suggesting that some PE_PGRS proteins are critical for everyday functions of the bacterium. Alternatively, variable expression levels of a number of other PE_PGRS genes in response to changing environmental conditions were observed. Published studies using RT-PCR and microarray techniques have also suggested that there are changes in the expression of a few PE_PGRS genes when altering growth conditions during culture of *M. tuberculosis* (1,

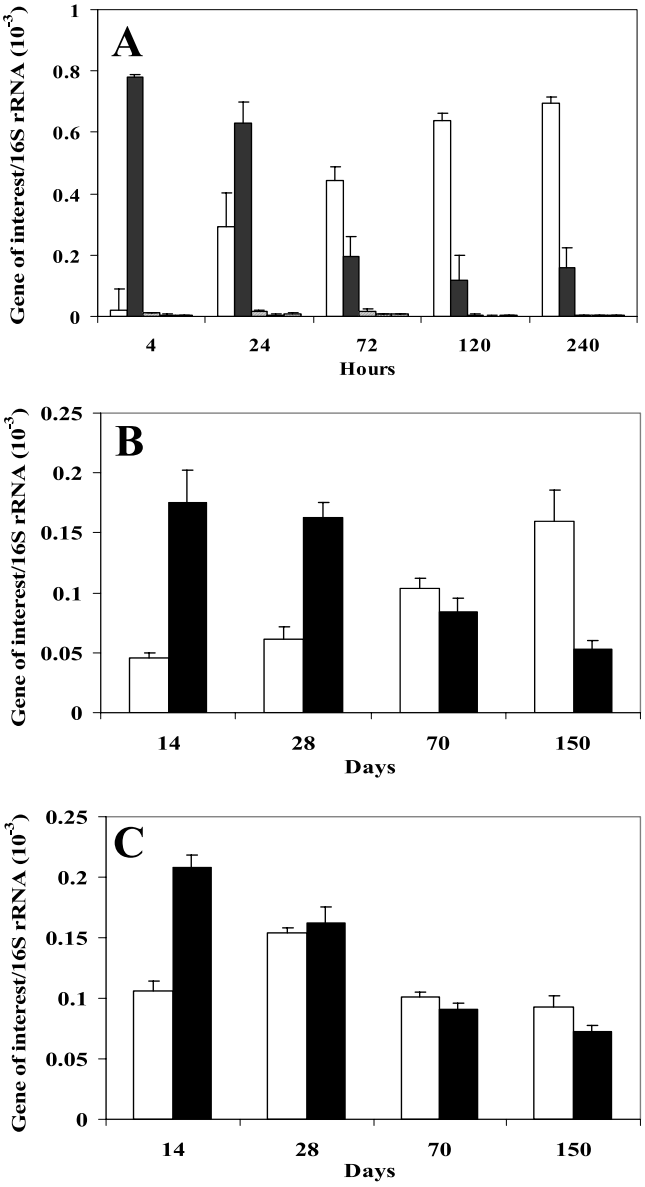

FIG. 4. Quantitative determination of PE_PGRS gene expression by *M. tuberculosis* residing within macrophages and mouse tissues. (A) BMMO cells were infected with *M. tuberculosis* strain Erdman, cells were harvested at different time points, and real-time RT-PCR was performed on the extracted RNA. The relative mRNA expression levels of genes PE_PGRS16 (open bars), PE_PGRS26 (filled bars), and PE_PGRS44, PE_PGRS51, and PE_PGRS55 (striped bars; some values are too small to be seen) compared to that of the 16S rRNA gene are shown over a time period of 240 h. (B and C) C57BL/6 mice (five mice per group) were aerogenically infected with *M. tuberculosis* Erdman, and RNA was extracted from spleens (B) and lungs (C) at various time points. Expression of PE_PGRS16 (open bars) and PE_PGRS26 (filled bars) was determined by real-time RT-PCR and is provided as a ratio normalized to the expression of the *M. tuberculosis*-specific 16S rRNA gene. Error bars show standard deviations.

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10, 12, 14, 19, 20, 23, 24). These findings and our studies suggest that specific regulatory mechanisms which control expression of certain PE_PGRS genes in response to different environmental signals could alter the composition as well as functional and antigenic properties of the mycobacterial cell wall, since there is evidence that certain PE_PGRS proteins are present at the cell surface (1, 7) and that they have a role in mediating mycobacterium-host cell interactions (4, 8, 9). This could have profound effects on how the mycobacterium is presented to the host immune system. Of particular interest in our studies was the finding that expression of PE_PGRS16 and PE_PGRS26 by *M. tuberculosis* persisting in mouse tissues was inversely regulated over several months of infection, which suggests that it may be advantageous for the pathogen to upregulate expression of PE_PGRS16 while down-regulating expression of PE_PGRS26. Further studies should focus on characterizing these two PE_PGRS proteins and determining if they have a particular role in helping the organism evade the host immune response and in latency. Additional studies may reveal that measuring inverse expression of PE_PGRS16 and PE_PGRS26 could serve as a "marker" for latent *M. tuberculosis* infection. In summary, the findings described here identify certain PE_PGRS genes of particular interest for further study and indicate that differential expression of certain PE_PGRS proteins may be an important factor in the immunopathogenesis of tuberculosis.

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