Hypoxia-responsive expression of *Mycobacterium tuberculosis* Rv3134c and devR promoters in *Mycobacterium smegmatis*

Mycobacterial persistence is a major hindrance in the treatment of tuberculosis. Persistence is thought to result from bacterial adaptation to the prevailing environment within tuberculous lesions and granulomas that are believed to be deficient in oxygen and nutrient supply (Wayne & Sohaskey, 2001). *In vitro* models of mycobacterial persistence have provided us with valuable insights into the physiological, structural, metabolic and molecular changes that accompany adaptation to oxygen limitation (Wayne & Sohaskey, 2001). A recent global analysis of expression revealed that the expression of more than 100 genes was altered when *Mycobacterium tuberculosis* was subjected briefly to defined hypoxic conditions (Sherman et al., 2001). Among the induced genes was the devR–devS two-component regulatory system. The genes encoding the DevR–DevS two-component system were first identified and partially characterized in *M. tuberculosis* (Dasgupta et al., 2000) and found to be well conserved in several mycobacteria including *Mycobacterium smegmatis* (Zahrt & Deretic, 2001; Tyagi & Sharma, 2002). *M. smegmatis* exhibits an adaptation response to oxygen limitation that is similar to that of *M. tuberculosis* (Dick et al., 1998). In fact, the molecular mechanisms involved in the dormancy response are likely conserved in the two species in so far as the involvement of the DevR–DevS genetic system is concerned (Mayuri et al., 2002). The slow growth rate and pathogenicity of *M. tuberculosis* often pose a hurdle for the study of its physiology. This has prompted the use of a fast-growing, non-pathogenic species, namely *M. smegmatis*, as a surrogate host. In view of the conservation between the two species of several aspects of the *in vitro* hypoxia response (Wayne & Sohaskey, 2001; Dick et al., 1998), the transcription machinery (Bashyam et al., 1996), several sigma factor genes (Tyagi & Sharma, 2002) and hypoxic induction of Rv3134c, devR and devS (Sherman et al., 2001; Mayuri et al., 2002), we assessed the suitability of *M. smegmatis* for studying the regulation of the *M. tuberculosis* devR–devS promoter.

Rv3134c, devR and devS are co-transcribed from the complementary strand of the *M. tuberculosis* genome. The coding regions of devR and devS overlap by 1 bp, and a 27 bp segment separates Rv3134c from devR (Dasgupta et al., 2000). We reasoned that sequences mapping upstream of devR and Rv3134c were likely to contain promoter(s) driving transcription of these genes. The promoter activity of this region was assessed by *lacZ* reporter assays in *M. smegmatis* cultures. Briefly, Rv3134c- and devR-upstream sequences were PCR-amplified and cloned in the integration-proficient vector pDK16 (Jain et al., 1997; A. K. Tyagi & D. Kaushal, unpublished data), to generate pDP3 and pDP2 (Fig. 1a). Both constructs supported a low β-galactosidase activity that was
Fig. 1. (a) Schematic representation of the devR–devS locus in M. tuberculosis. Solid black arrows represent the regions of the M. tuberculosis genome used for promoter studies. A 701 bp fragment (nucleotides 3500655–3501355; Cole et al., 1998) was PCR-amplified from M. tuberculosis DNA using primers R7 and R9c and cloned into pDK16 to generate pDP3. Similarly, a 1.45 kb fragment (nucleotides 3499904–3501355; Cole et al., 1998) was amplified using primers R7 and R3 and cloned to generate pDP2. (b) β-Galactosidase activity of M. smegmatis cultures carrying pDP2 and pDP3 grown under aerobic (▲, pDP2; ●, pDP3) and hypoxic (●, pDP2; □, pDP3) conditions; activities are the average of at least three independent experiments and are represented after subtraction of activities obtained with vector control. 'f' and 'd' refer to fading and decolorization of the redox indicator dye, methylene blue. (c) RT-PCR performed with RNA isolated from M. smegmatis cultures carrying pDP2. Primers R8 and R9c map upstream of the Rv3134c gene of M. tuberculosis. 23S rRNA transcripts were detected using primers mapping in the 23S rDNA gene. Lanes: 1, aerobic culture; 2, hypoxic culture; +, DNA positive control.

Consistently higher than that of the vector control in aerated cultures. Subsequently, the activity of putative P_Rv3134c and P_Rv3134c_devR promoters (in pDP3 and pDP2, respectively) was assessed in cultures grown in a sealed, static system where bacteria slowly settled to the bottom of the tube during growth causing oxygen depletion (Mayuri et al., 2002). Oxygen depletion was monitored via the fading and decolorization of methylene blue, which occurred by 24 and 48 h, respectively. Under these conditions, the various cultures grew in an exponential manner to a cell density of ~2 x 10^7 cells ml^-1 and then entered a plateau phase by day 6 or 7, which extended till the experiment was terminated on day 15. In contrast, under aerobic conditions, cells grew exponentially to an initial cell density of 3 x 10^6 to 1 x 10^8 cells ml^-1 and shifted thereafter to stationary phase. In cultures carrying pDP3 (P_Rv3134c promoter construct), a 3-3-fold induction in lacZ activity was noted just 24 h after the initiation of static culture. A decline in activity was noted thereafter till day 4; lacZ activity was then stably maintained at 1.5-fold of basal level till the end of the experiment. In cultures carrying pDP2 (P_Rv3134c_devR promoter construct), a 5-7-fold induction in lacZ activity was noted at 24 h, which was followed by a steady decline in activity till day 4; thereafter lacZ activity was maintained at 2-2-fold of basal activity till the end of the experiment (Fig. 1b). Comparison of the β-galactosidase activities on day 1 of hypoxic cultures carrying pDP2 versus pDP3 pointed to the dual promoter (P_Rv3134c_devR) being ~2-6-fold more active than the P_Rv3134c promoter. This indicated that this locus was driven by at least two hypoxia-responsive promoters: one mapping upstream of Rv3134c and the other upstream of devR. Induction of P_Rv3134c promoter activity was also confirmed by Reverse Transcriptase-PCR (RT-PCR) performed using RNA isolated from aerated and hypoxic cultures (day 2). RNA input in the reaction was first normalized by RT-PCR targeting 23S rDNA. M. tuberculosis-specific primers R8 and R9c, targeting the P_Rv3134c promoter region, were used to amplify a 236 bp fragment from M. smegmatis cultures carrying pDP2. An ~3-fold increase in transcript levels from this region was noted in hypoxic cultures versus aerated cultures (Fig. 1c).

The spurt in promoter activity under hypoxic conditions coincided with the fading of methylene blue in the control cultures, indicating that maximal induction occurred upon exposure of cultures to microaerophilic conditions and not when anaerobiosis was established. This suggests a requirement for DevR–DevS in the adaptation of the organism to gradually decreasing concentrations of oxygen. A recent study has shown DevR to be a key regulator required for adaptation of M. smegmatis to hypoxia (O’Ttoole et al., 2003).

The promoter-fusion experiments described here establish that the reported
hypoxia-dependent upregulation of Rv3134c, devR and devS is due to the induction of at least two M. tuberculosis promoters that are recognized and regulated in M. smegmatis. We believe M. smegmatis should prove useful for the analysis of the hypoxia response involving the devR–devS two-component system of M. tuberculosis.

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