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
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 \(~2 \times 10^7\) 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 \(3 \times 10^8\) to \(1 \times 10^9\) cells ml\(^{-1}\) and shifted thereafter to stationary phase. In cultures carrying pDP3 (P_{Rv3134c} promoter construct), a 3-3-fold induction in \(\textit{lacZ}\) activity was noted just 24 h after the initiation of static culture. A decline in activity was noted thereafter till day 4; \(\textit{lacZ}\) 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 \(\textit{lacZ}\) activity was noted at 24 h, which was followed by a steady decline in activity till day 4; thereafter \(\textit{lacZ}\) activity was maintained at 2-2-fold of basal activity till the end of the experiment (Fig. 1b). Comparison of the \(\beta\)-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. \textit{M. tuberculosis}-specific primers R8 and R9c, targeting the P_{Rv3134c} promoter region, were used to amplify a 263 bp fragment from \textit{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 \textit{M. smegmatis} to hypoxia (O’Toole 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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