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Differential expression of *dnaA* and *dosR* genes among members of the *Mycobacterium tuberculosis* complex under oxic and hypoxic conditions

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Summary. Major differences regarding the pathology and host immune response of the Beijing and Canettii genotypes of *Mycobacterium tuberculosis* have been reported; however, studies on the genetic expression of these genotypes during in vitro dormancy are scarce. This study examined the expression of five cell-cycle-related genes and two dormancy-related genes in *M. canettii, M. tuberculosis* H37Rv, and *M. tuberculosis* Beijing during the Wayne model of dormancy. The results showed that under hypoxic conditions the three tuberculosis genotypes were able to transcribe genes involved in DNA replication and cellular division. In addition, *dosR* was found to be up-regulated in *M. tuberculosis* Beijing during the exponential growth phase but down-regulated under hypoxic conditions. In this genotype, the replication-related gene *dnaA* was also strongly down-regulated. These latter two findings suggest that, compared to *M. tuberculosis* H37Rv and *M. canettii*, the Beijing genotype has a lower capacity to synthesize *dosR*, *hspX*, and *dnaA* mRNAs during in vitro dormancy. [**Int Microbiol** 2010; 13(1):9-13]

Keywords: *Mycobacterium tuberculosis* complex · differential gene expression · hypoxia · cell cycle

Introduction

Tuberculosis (TB), a disease caused by microorganisms belonging to the *Mycobacterium tuberculosis* complex (MTC), causes 9 million new infections and 2 million deaths each year [http://www.who.int/tb/country/global_tb_database/]. Recent epidemiological data suggest that the evolution of TB is strongly related to the MTC strain; for example, Lopez et al. [8], using the BALB/c mouse model of pulmonary TB, observed important differences in disease pathology and host immune response after infection with *M. tuberculosis* strains from the major genotypes found worldwide: *M. canettii*, *M. tuberculosis* H37Rv, and *M. tuberculosis* Beijing [8]. *M. canettii* infection induced a slowly progressive disease characterized by delayed bacterial multiplication, limited pneumonia, steadily increasing granuloma development, but no mortality. Conversely, Beijing infection consistently induced accelerated bacterial multiplication, early and massive pneumonia, and premature host death.

The Beijing/W lineage of *M. tuberculosis* (originally identified on the basis of strain IS*6110* fingerprinting and spoligotype patterns) has attracted considerable attention over the past decade because of its increased virulence and

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tendency to develop drug resistance [2]. On a global basis, Beijing strains account for approximately 10% of all TB cases [6,11]. This lineage of *M. tuberculosis* is dominant in Asia [2,16], and the spread of these strains has been documented worldwide [5]. The reasons for the apparent global extension of the Beijing strains are not yet understood but might include factors such as human migrations, selective pressure due to increased worldwide BCG vaccine coverage, and ineffective treatment of drug-resistant strains [11]. Little is known about the genetic expression of Beijing strains during the course of infection, especially during dormancy. Reed et al. [2007] reported up-regulation (50-fold) of the transcriptional factor DosR in Beijing strains compared to expression in *M. tuberculosis* H37Rv during exponential growth of the bacterium. Nevertheless, gene expression in Beijing and *M. canettii* strains during in vitro dormancy remains poorly understood.

In this study, the expression of five cell-cycle-related genes by the Beijing strain during the in vitro Wayne model of dormancy was quantified. The broader aim was to understand the physiological state of *M. tuberculosis* Beijing as a function of the bacterium's shift from an active to a persistent stage.

Materials and methods

Bacterial strains and growth conditions. Three strains of the MTC (*M. canettii* 17728, *M. tuberculosis* H37Rv, and *M. tuberculosis* Beijing 9501000) were grown in Dubos medium supplemented with 10% albumin-dextrose-catalase enrichment until an optical density (OD_{580}) of 0.2–0.4 was reached. The cultures were then subjected to hypoxia, essentially as described by Wayne and Hayes [20]. Each strain was selected according to the results obtained by López et al. in 2003 [8], in which it was demonstrated that *M. canettii* 17728 and *M. tuberculosis* Beijing 9501000 represent distinct genotype families. *M. tuberculosis* H37Rv was used as control.

RNA isolation and cDNA synthesis. Total RNA from each strain was isolated from cultures [14] in the exponential phase of growth and in non-replicative persistence 1 (NRP1, 4 days of exposure to hypoxia for *M. canettii* and 7 days for *M. tuberculosis* Beijing). cDNA was prepared using 1 μg of RNA, random hexamers (0.5 μg/μl), and AMV-RT (10 U/μl, reverse transcriptase of avian myeloblastosis virus; Promega).

Quantitative real-time PCR. Five cell-cycle (*dnaA, smc, parA, parB*, and *ftsZ*) and two dormancy-related (*hspX* and *dosR*) genes were quantified by real-time PCR using gene-specific primers (designed by the Primer Premier Designer program V.3.0 based on the genome sequence for *M. tuberculosis* H37Rv NC_000962). Primer sequences were: *dnaAF*: 5′-GCGACGTAGACGTGCTGTTG-3′ and *dnaAR*: 5′-GGCATTGTGCAAGGTGTTGA-3′; *smcF*: 5′-TGAACTGGATCAAGGCGAGGTC-3′ and *smcR*: 5′-CAACGCGGCCACAGTACG-3′; *ftsZF*: 5′-GCAGCGACTTGGGCTTGTTC-3′ and *ftsZR*: 5′-GGGTGAGCGGCGTCTTGTAC-3′; *parAF*: 5′-TCACCACCGTGATCCTTACCA-3′ and *parAR*:5′-CTTGATCGGCGAGCTTTGTC-3′; *parBF*: 5′-GCGTAAGCCGATTCAGATGCC-3′ and *parBR*: 5′-CCGACGCGAACTCCACCAC-3′; *dosRF*: 5′-CGCCTCGATGGTCTGGTG-3′ and *dosRR*: 5′-CACGATAGCGCGTAGGGTTG-3′; *hspXF*: 5′-AGCAGAAGGACTTCGACGGTC-3′ and *hspXR*: 5′-GTGCGAACGAAGGAACCGTA-3′; P1F 5′-CCTATGGATATCTATGGATGACCGAAC-3′ and P1R: 5′-GGCGACCCTGCCAGTCTAA-3′; 16SF: 5′-ATGACGGCCTTCGGGTTGTAA-3′ and 16SR: 5′-CGGCTGCTGGCACGTAGTTG-3′. Absolute mRNA transcript levels for target genes were normalized to the geometric mean of the 16S rRNA plus the P1 promoter of the *rrn* operon [9,15]. To analyze quantitative differences in gene expression among the strains, logarithmic graphics of the expression of each gene (reported as copies of the gene/ng RNA) were created. SYBR Green was used as fluorochrome (Molecular Probes) in the Rotor Gene 3000 system (Corbett Research). To validate quantitative real-time PCR assays and determine their efficiency, serial dilutions of genomic DNA of *M. tuberculosis* H37Rv were tested. Quantitation was carried out five times, using RNA isolated from three different batches obtained from each condition. The results were analyzed in a regression curve (calibration curve) considering that the regression value (r^2) must be close to 1 and the efficiency close to 2.

Statistical analyses. Two-way ANOVA and Turkey's multiple-comparison procedure were used to determine the significance of differences between gene expression of strains of the MTC under oxic and hypoxic conditions. SIGMASTAT version 3.5 was used to calculate P, with $P < 0.05$ considered significant.

Results and Discussion

The expression of cell-cycle genes by all three mycobacteria at exponential phase showed a similar pattern (Fig. 1A), suggesting that cell division by slow-growing mycobacteria, such as *M. canettii*, *M. tuberculosis* H37Rv, and *M. tuberculosis* Beijing, is tightly regulated. The most critical differences were observed in the expression of *smc*, which encodes the structural maintenance of chromosome protein SMC. The rate of expression of this gene in *M. canettii* and *M. tuberculosis* Beijing was similar and 5–5.3-fold lower than in *M. tuberculosis* H37Rv (Fig. 1A). Differences in the expression of *parA* and *parB* were expected, because each gene is regulated by distinct promoter regions, as reported by Casart et al. [3]. In the three species, *parB* expression was always higher than *parA* expression, in agreement with observations in the slow-grower *M. bovis* BCG, in which *parB* expression is also higher than that of *parA* along the mycobacterial growth curve [3].

Remarkable differences in the expression of the hypoxia genes *dosR* and *hspX* during exponential phase were found among the three strains. The results suggested that *dosR* expression was related to the level of virulence of the species (Fig. 1A); this gene was up-regulated in *M. tuberculosis* Beijing compared to its expression in *M. tuberculosis* H37Rv and *M. canettii* (17- and 195-fold, respectively) (Fig. 1A). Up-regulation of *dosR* was previously found during stationary phase and under other stress conditions, including starvation, in *M. tuberculosis* H37Rv. It has been hypothesized that the gene's early expression during exponential phase pre-

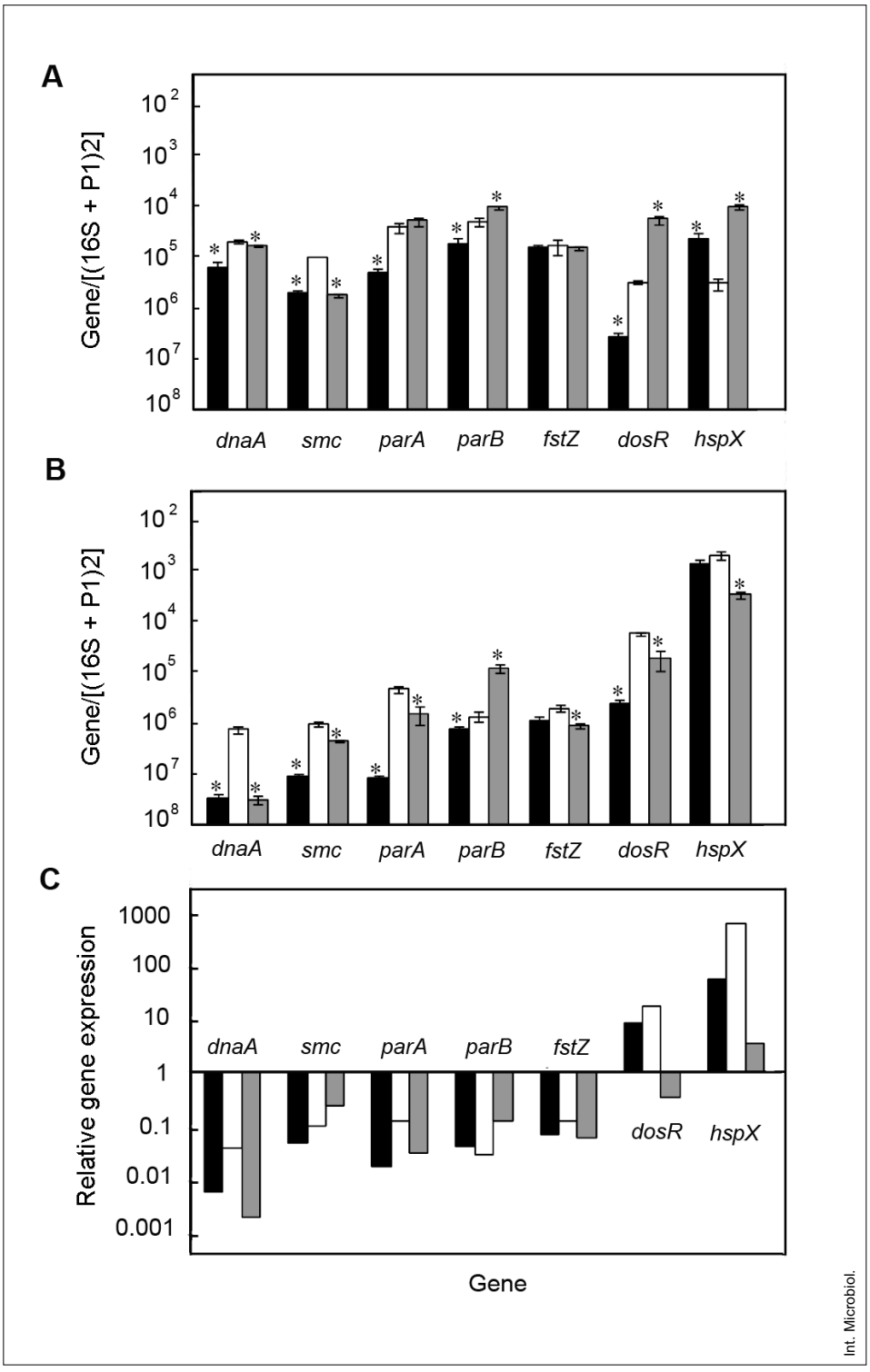

Fig. 1. Gene expression of *Mycobacterium canettii*, *M. tuberculosis* H37Rv and *M. tuberculosis* Beijing during the exponential phase and during hypoxia. (**A**) Gene expression during exponential phase. (**B**) Gene expression under hypoxic conditions. (**C**) Ratio of hypoxia/exponential phase expression. Black bars, *M. canettii*; white bars, *M. tuberculosis* H37Rv; grey bars, *M. tuberculosis* Beijing. Asterisks indicate statistically significant differences compared to *M. tuberculosis* H37Rv. (Three independent RNA samples were analyzed and mRNA levels were normalized to the mean of 16S rRNA plus P1 promotor.)

pares the bacterium for survival under hypoxia and other stress conditions [1,10,17,18]. Our results also confirm the hypothesis of Reed et al., i.e., that *dosR* is constitutively expressed in *M. tuberculosis* Beijing during the exponential growth phase [11].

Regarding *hspX* expression, this gene is apparently more active in *M. canettii* and *M. tuberculosis* Beijing than in *M. tuberculosis* H37Rv during exponential phase. In *M. tuberculosis* Beijing, *hspX* expression was 31-fold higher than in *M. tuberculosis* H37Rv. Therefore, expression of the gene did not seem to be related to strain pathogenicity. In fact, in the hypovirulent strain *M. canettii*, *hspX* expression was eight-fold higher than in *M. tuberculosis* H37Rv.

The expression of the same set of genes was also investigated during in vitro hypoxia (Fig. 1B). With respect to cell-cycle gene expression, the most relevant down-regulation was found in *dnaA*; the expression of this gene in *M. tuberculosis* Beijing was 24-fold lower than in *M. tuberculosis* H37Rv. Analysis of the expression of hypoxia genes in the three strains showed that, under this type of stress, *dosR* gene was up-regulated in *M. canettii* and *M. tuberculosis* H37Rv but down-regulated in *M. tuberculosis* Beijing (Fig. 1B). Similarly, the lowest rates of *hspX* expression during hypoxia were noted in *M. tuberculosis* Beijing (6-fold lower than in *M. tuberculosis* H37Rv), and the highest in *M. tuberculosis* H37Rv. This pattern was opposite to that observed during exponential phase, in which *M. tuberculosis* Beijing had the highest rate of expression and *M. tuberculosis* H37Rv the lowest.

In a more comprehensive approach, the ratio of normalized cDNA copies during in vitro dormancy and exponential phase was determined (Fig. 1C); our results showed a strong down-regulation of all cell-cycle genes in the three strains of *M. tuberculosis* analyzed. Furthermore, the ratio of expression of *dnaA* in hypoxia vs. exponential phase was 19-fold lower in *M. tuberculosis* Beijing than in *M. tuberculosis* H37Rv (Fig. 1C). This particular result suggested that, during hypoxia, *M. tuberculosis* Beijing is unable to synthesize mRNA involved in DNA replication at the same rate as during exponential phase. Therefore, *M. tuberculosis* Beijing might be unable to enter a persistent state, unlike *M. tuberculosis* H37Rv. This hypothesis is supported by the observation of Lopez et al. [8], who found that BALB/c mice infected with *M. tuberculosis* Beijing die earlier than control mice infected with the H37Rv strain, suggesting that *M. tuberculosis* Beijing does not enter a persistent state but instead rapidly kills its host. Despite the fact that genes controlling the cell cycle are down-regulated under hypoxic conditions in these three species of mycobacteria, our results imply that dormancy does not mean that DNA replication and bacterial division stop entirely, at least not at the transcriptional level.

DosR is a transcriptional regulator that mediates the response of *M. tuberculosis* to oxygen limitation [10,12,13]. Expression of the protein is up-regulated in *M. tuberculosis* H37Rv and *M. bovis* BCG both during stationary phase and during hypoxia [19]. An analysis of the relative expression of all the genes considered in this study (Fig. 1C) showed that, under hypoxic conditions, *dosR* was up-regulated in *M. tuberculosis* H37Rv and in *M. canettii* but down-regulated in *M. tuberculosis* Beijing (53- and 25-fold lower than in the two other strains, respectively). These results suggest that *M. tuberculosis* Beijing does not respond to hypoxic stress, in contrast to the other two *M. tuberculosis* species. This lack of response is consistent with pre-adaptation of this hypervirulent strain during exponential phase in order to survive at the onset of hypoxia.

The role of *hspX* during in vitro hypoxia is not yet completely understood [4]. In this study, *hspX* expression in *M. tuberculosis* Beijing during dormancy was 3.6-fold higher than during exponential growth. In contrast, in *M. tuberculosis* H37Rv, *hspX* was over-expressed 658-fold under the same conditions (Fig. 1C). Our results agree with those of Hu et al. [7], who suggested that *hspX* deletion leads to the selection of a hypervirulent phenotype.

In conclusion, we found that the expression in *M. tuberculosis* Beijing of replication-related genes was lower during in vitro hypoxia than during exponential growth. Our results indicate that strains of this genotype have a low capacity to synthesize transcripts that would allow persistence during in vitro dormancy. In addition, *M. tuberculosis* Beijing did not respond to hypoxic stress in the same way as *M. tuberculosis* H37Rv or *M. canettii*, implying that the Beijing strain is pre-adapted during exponential phase to survival during the onset of hypoxia.

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