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ORIGINAL ARTICLE

Overexpression of DosR in *Mycobacterium tuberculosis* does not affect aerobic replication in vitro or in murine macrophages

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Abstract *Mycobacterium tuberculosis* H37Rv, constitutively expressing a second copy of the transcriptional regulator DosR (*Rv3133c*) under control of the *hsp60* promoter, was compared to wild-type *M. tuberculosis* for in vitro expression of the target genes of the DosR dormancy regulon, for its in vitro growth characteristics in liquid 7H9 culture medium, and for its capacity to replicate in murine macrophages. Under aerobic conditions, *hsp60*-driven DosR significantly induced the expression of 39 out of 44 DosR regulon genes, as assessed by real time qPCR. Increased DosR regulon gene transcription in vitro did not modify the capacity of the strain to grow under axenic conditions nor to infect murine macrophages as compared to unmodified wild-type bacteria.

Keywords Tuberculosis · DosR · Latency · Macrophage

Introduction

Tuberculosis (TB) continues to be a leading infectious disease worldwide, causing nearly two million deaths per year, with an estimated one-third of the world population being latently infected with *Mycobacterium tuberculosis* (Mtb), according to the World Health Organization. The existing live vaccine, *Mycobacterium bovis* BCG, shows a variable efficacy in most developing countries where the major burden of disease occurs (Andersen et al. 2004; Kaufmann 2007). Although this vaccine is effective against extrapulmonary forms of TB, which affect mostly children, the vaccine confers only variable protection against the pulmonary form of the disease in young adults, which develops, in many cases, as the result of the reactivation of a latent TB infection (Demissie et al. 2006; Young et al. 2008). In vitro cultures of 'stressed' Mtb, grown under conditions of nutrient starvation (Betts et al. 2002), hypoxia (Sherman et al. 2001; Rosenkrands et al. 2002), or low pH (Fisher et al. 2002) are thought to mimic at least in part the in vivo conditions faced by dormant tubercle bacilli in lung granulomas. Best studied so far in this respect have been the 48 gene products of the dormancy DosR regulon, which is controlled by the two-component sensor and transcriptional regulator DosS/DosR (Rv3132c-3133c) (Park et al. 2003; Voskuil et al. 2003), and which is strongly expressed under conditions of hypoxia and non-replicating persistence (NRP) in vitro. Similar transcriptional adaptations of Mtb have also been reported in models of IFN-+γ activation of mouse macrophages (Schnappinger et al. 2003) and in vivo in persistently infected mouse lungs (Shi et al. 2003). BCG vaccination induces only weak responses against the prototype latency antigen HspX (α-crystallin, Rv2031c) (Vekemans et al. 2004; Geluk et al. 2007) and against other antigens encoded by the DosR regulon (Lin et al. 2007). It has been hypothesized that this lack of induction of latency antigen-specific immune responses in BCG vaccinees is responsible, at least in part, for the low protection that BCG confers against reactivation TB.

So far, the role of the DosR regulon in Mtb virulence is not clear and conflicting results about its role have been reported

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in experimental animal models (Converse et al. 2009; Bretl et al. 2012).

In this work, we have used a different approach to further analyze the relevance of the *dosRS* system for tuberculosis infection, by driving DosR (*Rv3133c*) expression under the control of a constitutive promoter (p*hsp60*) so that it is produced even under aerobic conditions, and evaluate its impact on in vitro growth and during macrophage infection.

Materials and methods

Bacterial strains and media The laboratory strain H37Rv and its isogenic derivatives were cultured using Middlebrook 7H9 media (BD, Franklin Lakes, NJ, USA), containing 10 % Middlebrook ADC enrichment (BD, Franklin Lakes, NJ, USA), 0.5 % glycerol (Fisher Scientific, Pittsburgh, PA, USA) and 0.05 % Tween 80 (Sigma Aldrich, St. Louis, MO, USA), or 7H10 agar (BD, Franklin Lakes, NJ, USA) containing 10 % OADC and 0.5 % glycerol. *E. coli* DH5α or TOP-10 (Invitrogen, Carlsbad, CA, USA) were used as hosts when constructing recombinant plasmids and were cultured using LB agar plates or broth. Kanamycin (25 or 50 μg/ml, Sigma Aldrich, St. Louis, MO, USA) was used when selecting for recombinant strains (*M. tuberculosis*) or plasmids (*E. coli*), respectively. When constructing growth curves, the absorbance at optical density (O.D.) at 600 nm was recorded on a daily basis. For Mtb::DosR construction, we used pMF361DosR, obtained in a previous study (Flores Valdez and Schoolnik 2010). Briefly, the wild-type *Mycobacterium tuberculosis* (Mtb) *dosR* gene was amplified from Mtb strain 1254 using Platinum Pfx High Fidelity Supermix (Invitrogen) and primers devR-FPvu2 (50GTGCAGCTGTCATGGTAA AG GTCTTCTTGGTCG-3) and devR-RHd3 (50-ACTAAG CTTCCTGTTGTC ATGGT CCATCACCG-3). The PCR product was cloned into pCR4, using a TOPO cloning system (Invitrogen), and then a PvuII/HindIII fragment was subcloned into pMV361, thereby creating pMF361dosR, which was transformed into MtbH37Rv by electroporation.

For the macrophage infection experiments, wild-type Mtb H37Rv and Mtb::DosR were grown for 2 weeks as a surface pellicle on synthetic Sauton medium (De Bruyn et al. 1987). Bacteria were harvested, homogenized by ball mill and aliquots were frozen at −80 °C in 20 % glycerol until use.

Transcriptional analysis cDNA was generated using 10 ng of total RNA with Maxima reverse transcriptase (Fermentas) using random hexamers and following the instructions provided by the manufacturer. A 15-cycle PCR amplification was performed with Advantage2 polymerase (Clontech). Generated cDNAs were amplified in hot start multiplex RT-PCR with a set of nested RTF-RTR TB gene-specific primers under conditions controlling for linearity of PCR amplification (Dolganov et al. 2001). Each PCR contained 3 μl of the initial 20 μl cDNA reaction and specific gene primers at 5 pM each. Reaction conditions were 95 ºC for 1 min, followed by 15 cycles of 95ºC for 30 s, 60ºC for 20 s, and 68ºC for 1 min.

Quantitative analysis of M. tuberculosis gene expression using two-step multiplex real-time RT-PCR PCR products were quantified in individual real-time PCR reactions using gene-specific 6FAM/3'BHQ-labeled TaqMan probes and primers designed with PrimerExpress3.0 (Applied Biosystems). Real-time PCR was performed using 0.07 μl amplified cDNA per gene, 1x Taqman Universal Master mix (Roche), and Taqman probes (http://genes.stanford.edu and http://www.tbdb.org/rtpcrData.shtml.) and run on the Lightcycler 480 (Roche). Real-time PCR conditions were: 95ºC for 5 min, followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s, and 40ºC for 30 s. Raw data were expressed as the number of PCR cycles needed to reach a detection threshold value (Ct) that is inversely proportional to the exponent of transcript abundance. Use of multiple internal controls allowed accurate quantification of transcripts in each sample across a board of multiple specimens. Originally, this method was developed for detection of low-level transcripts in small clinical specimens (Dolganov et al. 2001) and then adapted to *M. tuberculosis*-infected lesions. Marc Coram (Department of Biostatistics, Stanford) developed a practical and simple method for normalizing *M. tuberculosis* expression using multiple internal reference genes that agrees with GeNorm (Vandesompele et al. 2002) and is applicable for the analysis comparing samples with 200 vs. 2,000 genes measured. In this approach, the internal reference genes are those that showed the more conserved expression value among the different samples, i.e., genes whose transcription is not affected by experimental conditions and/or genes being deleted or inserted in recombinant strains. Therefore, these internal reference genes might be different in a study-to-study basis. Oligo sequences are available at http://genes.stanford.edu/oli/index.php.

In vitro replication of Mtb WT and Mtb::DosR in Mf4/4 macrophages

Immortalized Mf4/4 macrophages (Desmedt et al. 1998) were seeded in 6-well plates at 10^6 cells/2 ml in RPMI-1640 medium supplemented with 10 % FCS, penicillin, fungizone and $5x10^{-5}$ M β mercapto-ethanol and left to adhere for 1 h in a humidified CO_2 incubator at 37 °C. Next, cells were infected in triplicate for 3 h with Mtb WT or Mtb:DosR bacteria, vigorously dispersed by repeated needle aspiration, at a multiplicity of infection (m.o.i.) of 1. Cells were washed three times with 2 ml of complete RPMI-1640 medium to eliminate non-phagocytosed bacteria, and finally 5 ml of complete RPMI-1640 medium was added to each well. After 3 h, 1, 3,

and 5 days of incubation, wells were washed three times with 2 ml Dulbecco's phosphate buffered saline (DPBS, Lonza), and next 1 ml of DPBS + 1 % Triton X-100 Detergent (Calbiochem) was added for 15 min to lyse the macrophages. The cell lysate was collected in a 15 ml Falcon tube and wells were washed three times with 2 ml of DPBS. Total cell lysate was topped up to 10 ml, and 1-ml aliquots were supplemented with 30 % glycerol and stored at −80 °C until CFU plating.

Analysis of bacterial replication in Mf4/4 macrophages Serial dilutions of macrophage lysate were plated on 7H11 Middlebrook agar supplemented with 10 % OADC. Petri dishes were kept sealed in plastic bags, incubated at 37 °C and bacterial colonies were enumerated visually after 3 weeks. Numbers of bacteria are expressed as mean +/− SD $\log_{10}$ CFU/well (tested in triplicate).

Results

DosR expression under the control of a constitutive promoter does not disturb in vitro growth properties of M. tuberculosis H37Rv Oxygen depletion is an in vitro stimulus that induces *dosR* expression with concomitant upregulation of a 48-gene-set known as the DosR regulon (Sherman et al. 2001; Voskuil et al. 2003). A gradual depletion of oxygen leads to an in vitro non-replicating persistence (NRP) state characterized by bacteriostasis and metabolic, chromosomal, and structural changes of the dormant bacteria (Wayne and Sohaskey 2001). As growth arrest occurs coincident with *dosR* (*Rv3133c*) gene induction, this suggests either its direct participation or that of its downstream targets as responsible for limiting replication. To test this idea, we first assessed whether expressing *dosR* from the constitutive promoter of *hsp60* (Stover et al. 1991) would affect the growth of Mtb under aerobic conditions, a situation where we dissociate DosR production from response to oxygen limitation. As can be seen in Fig. 1a, under standard, shaking (100 rpm) aerobic in vitro conditions, the parental Mtb WT and its isogenic derivative containing the *hsp60*-driven *dosR* gene (Mtb:: DosR) showed identical growth kinetics. Likewise, growth under static conditions (Fig. 1b) was comparable for both strains. The doubling time during the log phase was determined using a non-linear exponential growth model in Statgraphics Centurion XVI. Data from days 0, 1, 2, and 3 were used in the calculation. In the shaking condition, the doubling time was 1.64 days for Mtb

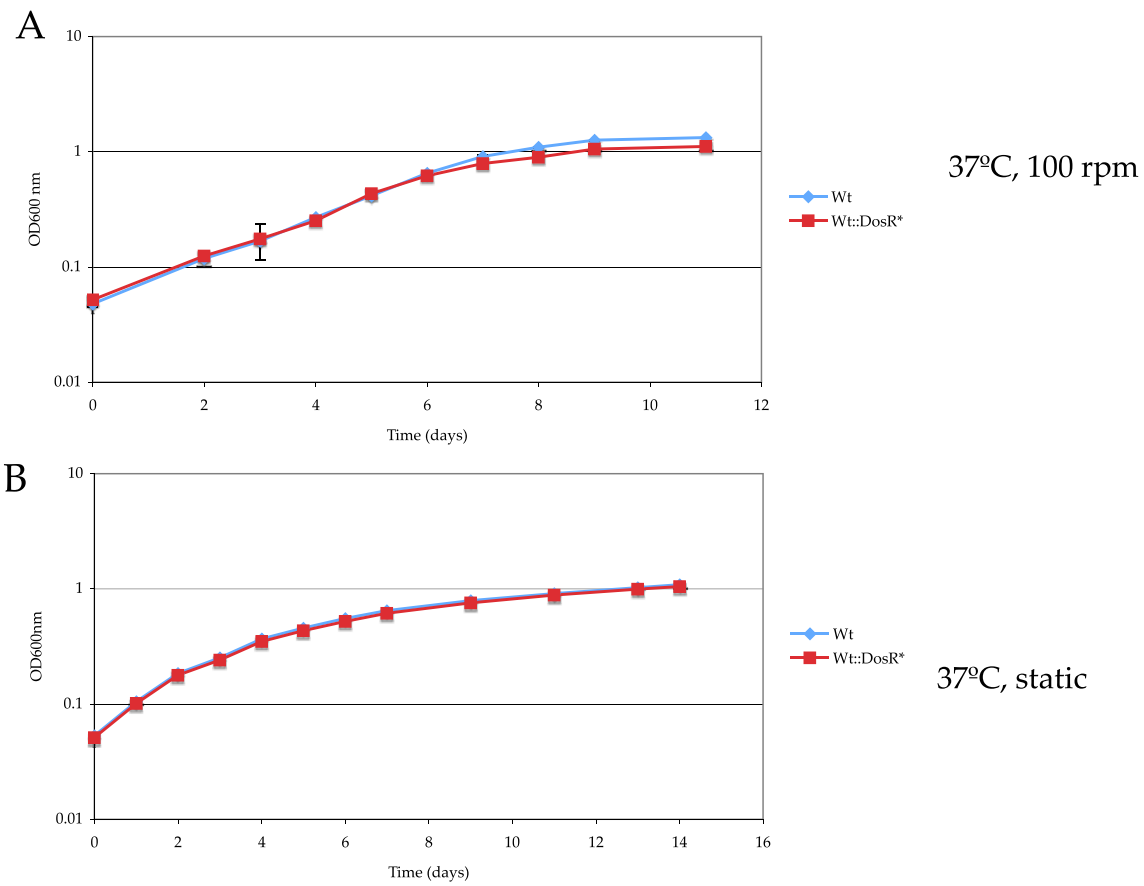

Fig. 1 Constitutive expression of *dosR* in *M. tuberculosis* H37Rv or in *M. tuberculosis* H37Rv does not affect in vitro growth kinetics. Strains were grown under: (**a**) shaken (100 rpm), 37ºC or (**b**) static (37ºC, 5 % CO_2) conditions

Table 1 Constitutive DosR expression in *M. tuberculosis* H37Rv results in activation of the DosR regulon under aerobic conditions

Rv number	Gene	Protein function	*Mtb*:*DosR*/*Mtb WT*	*T* test p-value
Rv0079		CHP	8.06	0.76016
Rv0080		CHP	90.82	0.47172
Rv0081		Probable transcriptional regulatory protein	21.71	0.17005
Rv0569		CHP	12.77	0.00025
Rv0570	*nrdZ*	Ribonucleotide reductase	17.51	0.01118
Rv0571c		CHP	6.96	0.00003
Rv0572c		CHP	10.85	0.00048
Rv0574c		CHP	2.51	0.009
Rv1733c		Probable conserved transmembrane protein	**202.95**	0.00044
Rv1734c		CHP	91.77	0.0003
Rv1736c	*narX*	Fused nitrate reductase	174.25	0.00016
Rv1737c	*narK2*	Nitrate extrusion protein	**271.54**	0.00105
Rv1738		CHP	**765.36**	0.00063
Rv1812c		Probable dehydrogenase	4.82	0.00434
Rv1813c		CHP	**310.83**	0.00062
Rv1996		CHP	47.34	0.00336
Rv1997	*ctpF*	Cation transport ATPase	58.28	0.00313
Rv2003c		CHP	13.69	0.00039
Rv2004c		CHP	22.71	0.00022
Rv2005c		CHP	51.8	0.00048
Rv2007c	*fdxA*	ferredoxin	**879.17**	0.0004
Rv2028c		CHP	157.59	0.0005
Rv2029c	*pfkB*	Phosphofructokinase II	177.29	0.00082
Rv2030c		CHP	**1573.76**	0.00005
Rv2031c	*hspX*, *acr*	**α-crystallin**	**1858.60**	0.0006
Rv2032	*acg*	CHP	124.07	0.00051
Rv2623		CHP TB 31.7	**307.62**	0.00033
Rv2624c		CHP	116.16	0.00059
Rv2625c		Transmembrane alanine and leucine rich protein	**292.04**	0.00102
Rv2626c		CHP	**1120.56**	0.00023
Rv2627c		CHP	**1164.10**	0.00027
Rv2628		HP	6.41	0.00991
Rv2629		CHP	32.45	0.00222
Rv2630		HP	23.75	0.00069
Rv2631		CHP	1.07	0.76304
Rv3126c		HP	1.98	0.07733
Rv3127		CHP	**362.04**	0.00101
Rv3128c		CHP	**617.37**	0.00029
Rv3129		CHP	131.6	0.00059
Rv3130c		triacylglycerol synthase	**942.27**	0.0005
Rv3131		CHP	133.44	0.00545
Rv3132c	*dosS*	Sensor histidine kinase	18.44	0.00486
Rv3133c	*dosR*	2-comp. Response regulator	26.91	0.00128
Rv3134c		CHP	68.36	0.00001
Rv0117	*oxyS*	Oxidative stress response regulatory protein	1.02	0.68409
Rv0166	*fadD5*	Fatty acid CoA synthase	0.96	0.83854
Rv0287	*esxG*	Esat-6 like protein	1.08	0.69576
Rv0288	*esxH*	CFP-7 (Protein 10.4)	0.9	0.10022
Rv0291	*mycP3*	Membrane-anchored mycosin MYCP3	1.07	0.76814

Table 1 (continued)

Rv number	Gene	Protein function	*Mtb*:*DosR*/*Mtb WT*	*T* test p-value
Rv0456c	*echA2*	Enoyl-CoA hydratase	0.96	0.61589
Rv0873	*fadE10*	Acyl-CoA dehydrogenase	1.18	0.71566
Rv0971c	*echA7*	Enoyl-CoA hydratase	0.93	0.63515
Rv0972c	*fadE12*	Acyl-CoA dehydrogenase	1.17	0.41122
Rv0973c	*accA2*	Acetyl/propionyl-CoA carboxylase	1.09	0.71979
Rv1037c	*esxI*	Esat-6-like protein	1.14	0.25013
Rv1038c	*esxJ*	Esat-6-like protein	1	0.98345
Rv1058	*fadD14*	Fatty acid-CoA ligase	0.9	0.42265
Rv1266c	*pknH*	Serine/protein kinase H	0.9	0.10834
Rv1915	*aceAa*	Isocitrate lyase	0.96	0.71513
Rv1926c	*mpt63*	Immunogenic protein MPT63	0.98	0.90609
Rv1980c	*mpt64*	Immunogenic protein MPT64	0.97	0.66152
Rv2346c	*esxO*	Esat-6-like protein	1.11	0.47498
Rv2347c	*esxP*	Esat-6-like protein	0.95	0.63349
Rv2500c	*fadE19*	Acyl-CoA dehydrogenase	0.95	0.50127
Rv3052c	*accD1*	Acetyl/propionyl-CoA carboxylase	1	1
Rv3763	*lpqH*	19 kD lipoprotein	1.14	0.59024
Rv3874	*esxB*	CFP-10	0.9	0.09858
Rv3875	*esxA*	ESAT-6	0.98	0.67327

Quantitative PCR transcriptional profiling on extracts of bacteria grown under aerobic (non-inducing) growth conditions in 7H9 liquid medium
Genes for which expression was increased more than 200-fold are indicated in bold

WT and 1.69 days for Mtb:DosR. In the standing condition, the doubling time was 1.29 days for Mtb WT and 1.30 days for Mtb:: DosR. The difference in doubling time was statistically significant between conditions ($P<0.01$) but was not statistically significant ($P>0.1$) between strains. However, for well-aerated, shaken cultures (Fig. 1a) from day 7 to day 11 (stationary phase), a slight difference was evident. After double tailed-*T* test performed with the OD values of each strain we determined such difference to be statistically significant (*P* values of 0.03, 0.008, 0.04, and 0.02 for days 7, 8, 9, and 11, respectively). No difference was observed for static cultures (Fig. 1b)

Production of DosR from the hsp60 constitutive promoter is sufficient to induce expression of DosR regulon genes under aerobic conditions. We next analyzed the expression of 44 DosR regulon genes under the conditions mentioned above by real-time quantitative PCR. We wanted to confirm whether the chromosomally integrated, p*hsp60*-driven DosR would be active or not under non-inducing conditions. As can be seen, 39 out of the 44 DosR regulon genes tested showed induction under aerobic conditions that was statistically different from expression in non-transformed, wild-type Mtb, thus demonstrating that expression from the constitutive promoter was sufficient to drive expression of most DosR-controlled genes in Mtb H37Rv (Table 1). For these 39 genes, expression levels in Mtb::DosR were at least two-fold higher than those observed in the parental, non-transformed strain. A number of other genes with diverse functions, including ESAT6-family members, some involved in lipid metabolism (*fad* and *echA* genes), and some encoding for known antigenic components (lpqH [19 kD lipoprotein], *Rv1926c* [Mpt63], *Rv 1980c* [Mpt64]) showed no change between strains (Table 1), confirming that they are not regulated by DosR and can be used as internal references. DosR regulon genes showing a non-statistically significant difference were *Rv0079*, *Rv0080*, *Rv0081*, *Rv2631*, and *Rv3126c* (although close to significance, $P=0.07$)

Constitutive expression of dosR by Mtb does not affect in vitro growth in Mf4/4 macrophages. Finally, we compared the bacterial replication of the two strains in murine Mf4/4 macrophages. Mtb::DosR showed the same growth characteristics in infected Mf4/4 macrophages as parental Mtb WT (Fig. 2). Thus, the number of bacteria of both isolates increased more than tenfold during the first day of culture and remained more or less stable during the next 4 days. At day 1 of culture, the number of Mtb::DosR bacteria was slightly lower than the number of Mtb WT, but at the three other time points tested, the CFU numbers were statistically not different between the two isolates

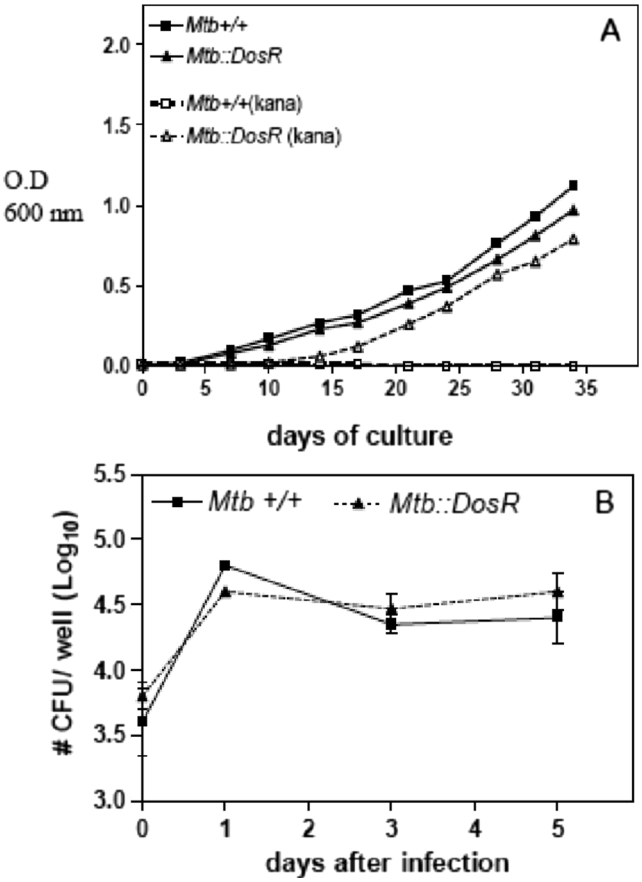

Fig. 2 Constitutive expression of *dosR* in *M. tuberculosis* H37Rv does not affect its in vitro growth in Mf4/4 macrophages. (**a**) Kanamycin at 50 μg/ml was added to in vitro cultures to show that it has selective pressure against wild-type *M. tuberculosis* (Mtb +/+). (**b**) Mf4/4 macrophages were infected at m.o.i. of 1 with Mtb WT or Mtb:DosR and monitored for intracellular bacterial replication for 5 days. Numbers of bacteria are expressed as mean +/− SD log10 CFU/well (tested in triplicate)

Discussion

The DosR (*Rv3133c*) transcriptional regulator has an instrumental role in promoting mycobacterial adaptation and survival to oxygen shift-down and anaerobiosis (Wayne and Sohaskey 2001). Some of its regulated proteins are proposed to serve as specific biomarkers for latent infection (Demissie et al. 2006), albeit new candidate molecules have also started to emerge (Schuck et al. 2009). In order to further explore the in vivo relevance of DosR, we constructed an Mtb strain expressing *dosR* under the control of the constitutive *hsp60* promoter.

During in vitro adaptation to low oxygen, Mtb and *M. bovis* BCG enter a state of non-replicating persistence, which coincides in time with DosR expression. This association suggests that expression of *dosR* or the DosR regulon it regulates might be hindering replication. Therefore, we first investigated whether the low oxygen-induced, DosR-associated non-replicating phenotype would be altered when DosR was expressed from the *hsp60* promoter during aerobic growth. We analyzed the in vitro growth of Mtb::DosR under aerobic, static and shaken conditions and found that *Mtb*::*DosR* replicated at the same rate as wild-type Mtb during the logarithmic phase of growth (Fig. 1a), with a slight, statistically significant delay occurring in Mtb:DosR during the stationary phase of growth (Fig. 1b). Recently, using an anhydrotetracycline-inducible promoter, it was shown that *dosR* did not affect growth under aerobic conditions while induced most of the genes controlled by this transcriptional regulator (Minch et al. 2012). Therefore, both studies are in agreement in showing that expression of *dosR* itself does not seem to be responsible for the known non-replicating persistent phenotype that is induced in Mtb by hypoxia. Rather, hypoxia likely causes growth arrest by a mechanism distinct from DosR regulon induction. Likewise, intracellular replication in murine Mf4/4 macrophages was similar for Mtb::DosR and wild-type Mtb.

We demonstrated that DosR produced during aerobic conditions was able to induce its regulon, as we observed significant induction of 39/44 genes in Mtb::DosR compared to the parental, not-modified wild-type strain (Table 1). Among the highest upregulated genes in Mtb::DosR, we found *Rv2031c* encoding the prototype latency antigen HspX, *Rv2626c*, and *Rv2627c*. DosR regulon genes showing a non-statistically significant difference were the dormancy associated translation inhibitor encoded by *Rv0079* (Kumar et al. 2012), the antigenic protein encoded by *Rv0080*, which showed stronger responses in Japanese patients with active tuberculosis than latent infection (Hozumi et al. 2013), the transcriptional regulator and proposed as regulatory hub encoded by *Rv0081* (Galagan *et al.* 2013), the hypothetical protein encoded by *Rv2631*, and the hypothetical protein encoded by *Rv3126c* (although close to significance, $P=0.07$). From these five genes, *Rv0079*, *Rv0080*, and *Rv0081* showed 8-, 90.8-, and 21.7-fold difference with respect to Mtb harboring only the chromosomal *dosR* copy (Table 1), so it could well be that technical variability present in our samples, perhaps because transcripts of these genes were particularly labile in some experiments and not in others, resulting in the lack of statistical significance. As for *Rv2631*, in one study this gene showed induction values of 3.4±2.1 under hypoxia (Park et al. 2003) while it showed a similar, 1.6-fold change value under the same condition, and it was only upon entering dormancy that it showed an increased, sixfold change (Voskuil et al. 2003); therefore, suggesting we might have missed additional stimuli to see this genes upregulated in our Mtb::DosR strain, or that the intracellular DosR concentration needed to induce this gene did not reach critical concentration as has been shown elsewhere (Majumdar et al. 2012). Regarding *Rv3126c*, this gene showed a very modest 1.7±0.7 induction under hypoxia (Park et al. 2003), but surprisingly this value went up to a 23-fold change under the same condition (Voskuil et al. 2003). We frankly found these differences rather difficult to explain,

given that these results came from different reports where several authors participated in both works.

In summary, constitutive expression of DosR can be accomplished in *M. tuberculosis* H37Rv with significant induction of 39 out of 44 DosR regulon genes evaluated, whereas it has no effect on aerobic growth and with no effect on replication within murine macrophages. It would be interesting to evaluate this strain in animal models, or during NRP adaptation, to further gain insight into the relevance of DosR for mycobacteria.

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