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DosR-regulon genes induction in *Mycobacterium bovis* BCG under aerobic conditions

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SUMMARY

In this report we demonstrated that under aerobic conditions, *Mycobacterium bovis* BCG expressing an *hsp60*-driven second copy of the hypoxia-related transcriptional regulator DosR increased 2-fold or greater the expression of 38 out of the 48 genes belonging to the DosR regulon, including the latency antigens *Rv1733c*, *Rv2029*, *Rv2627*, and *Rv2628*. Expression of DosR under these conditions slightly delayed *in vitro* growth, but did not promote a non-replicating state as opposed to microaerobic and hypoxic adaptation. Our results suggest BCG producing DosR can be cultured under standard *in vitro* conditions, allowing evaluation of this strain as a latency-specific vaccine candidate.

Keywords

Tuberculosis; Latency; Vaccine; BCG

1. Methods, results and discussion

In Mycobacteria, oxygen depletion is an *in vitro* stimulus that induces expression of the transcriptional regulator DosR, resulting in up-regulation of a 48 gene-set known as the DosR-regulon.[1,2] 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.[3] As growth arrest occurs coincidentally to DosR *(Rv3133c)* induction[4]; it suggests that DosR itself or its downstream targets could be at least partially responsible for limiting replication. We first assessed whether expressing a single copy of this gene from the strong promoter of *hsp60*,[5] integrated into the *attB* site, would affect the growth of *Mycobacterium bovis* BCG SSI 1331 when cultured in 7H9 media supplemented with albumin-dextrose-catalase and 0.05% Tween 80, grown at 100 rpm and 37 °C (hereafter referred as to aerobic conditions). For this, 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 (5′GTGCAGCTGTCATGGTAAAG GTCTTCTTGGTCG-3) and devR-RHd3 (5′-

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ACTAAGCTTCCTGTTGTC ATGGT CCATCACCG-3). The resulting PCR product was cloned into pCR4, using a TOPO cloning system (Invitrogen), and then a PvuII/HindIII fragment was subcloned into pMV361,[6] thereby creating a plasmid designated pMF361dosR that was transformed into BCG by electroporation to produce BCG::DosR. As can be seen in Figure 1A, under aerobic conditions, BCG::DosR showed a delayed growth curve compared to the parental strain (BCG), although it did not enter into a non-replicating persistence state as opposed to hypoxic cultures.[3] The presence of the empty vector, pMV361 (BCG::pMV361) did not result in growth perturbation. Conversely, when strains where cultured at 37 °C but allowed to settle undisturbed, thereby creating an oxygen gradient in the population, growth curves looked almost identical, regardless of the presence of vector or DosR copy number (Figure 1B). DosR induction produces metabolic changes, which might require *M. tuberculosis*-specific genes to allow bacteria to continue multiplying should oxygen be present, as a *M. tuberculosis*::DosR strain was not affected in its growth (Flores-Valdez *et al*, submitted), as opposed to BCG (this study). One such change allowing adaptation to microaerophilic conditions is expression of a DosR-controlled system to utilize the alternate electron acceptor nitrate, where differences occur between *M. tuberculosis* and *M. bovis* wild type and BCG.[7,8] In fact, regulation of these genes has been shown to be dramatically different between *M. tuberculosis* and *M. bovis* BCG.[9] Particularly, a single nucleotide change in the −10 promoter region of the narK2X operon has been shown to be responsible for inactivation of these genes in *M. bovis* wild type and BCG.[10]

When cells reached OD600 nm of 0.2, and 0.4, aliquots of 30, and 10 ml, respectively, were taken and centrifuged 3 min at 3000 *g* at room temperature. Then the supernatant was discarded, and the cell pellets were immediately frozen by immersion into dry ice. RNA was later isolated from cell pellets using Trizol and chloroform, followed by ammonium acetate/ isopropanol precipitation, then treated with Turbo DNase and Qiagen RNEasy mini columns purification. Afterwards, using transcriptional profiling by microarrays, we compared the expression of DosR-regulon genes at different points of the growth curve of BCG::DosR and BCG::pMV361 to the parental BCG strain. All data was scored for statistical significance by using significance analysis of microarrays (SAM)[11] with a false discovery rate (FDR) of 0%, performed on genes with no flag in the spotted arrays and whose regression correlation was 0.6. Whether DosR needed a hypoxic environment to be active or whether the protein was oxygen-labile were open questions. Results shown in Table 1 validated that *hsp60*-driven DosR was functional, as BCG:: DosR showed 2-fold or higher induction of 38 and 35 DosR-regulon genes during early-log ($OD_{600\ nm}$ 0.2) and mid-log phase ($OD_{600\ nm}$ 0.4) cultures respectively. The presence of the empty vector did not induce any DosR-regulon gene (Table 1). The slight variation in expression for some genes across the growth phases assessed could be explained if altered mRNA stability for some transcripts occur under aerobic conditions, or perhaps, as the culture progresses, some genes gained induction in BCG wild type used as the reference, solely due to bacterial clumping. Clumping in the BCG strain we used in this study could have somewhat contributed to induction of some DosR-regulon genes. However, when we compared by transcriptional profile BCG::DosR to a *M. tuberculosis* H37Rv devoid of the *Rv3134-dosRS* operon (Table 2), 25 out of 49 genes assayed showed no significant change. In fact, taking HspX as an example for expression ratio calculation, BCG::DosR/BCG showed almost a 36-fold change, while BCG::DosR/

H37RvΔ*Rv3134c-dosRS* resulted in almost a 31-fold change, which results only in a 15% increase. Therefore we do not believe that clumping is highly relevant for HspX increased production. Variation in expression of other genes might be the consequence of different genetic backgrounds.

We consider induction of DosR-regulon genes in BCG::DosR being an artifact upon cell pelleting producing localized hypoxia unlikely. Even though we did not evaluate transcription rate or RNA degradation, nor are we aware of a similar study conducted in 7H9 media, we base our argument on the following: (i) The reported RNA synthesis rate for H37Rv in Sauton media[12] is between 4 and 10 nt/s (when doubling time is 10 h, in our case it is closer to 20 h). This means that if RNA synthesis occurs at a similar rate during centrifugation, for *hspX* (435 nt) transcription should take between 108.75 and 43.5 s. Therefore, during the 3 min period of time of centrifugation that we used, a change between 1.6 and 4.1-fold could occur to its mRNA, also assuming transcription efficiency to be 100% under centrifugation. (ii) If we were to assume transcription induction occurs during centrifugation, we could also think of transcript decay as occurring too, thus raising speculation as to the effect of spinning down cells by centrifugation and its very complex impact on particular mRNAs transcription, stability, and degradation.

An enhanced activity of DosR has been suggested for Beijing strains.[13] Interestingly, the latency antigens *Rv1733c*, *Rv2029*, *Rv2627*, and *Rv2628*[14] were among genes induced in BCG::DosR, thus suggesting it could be evaluated as a potential new, latency-specific vaccine candidate that can be massively produced under aerobic conditions, perhaps with subtle control of culture conditions.

DosR requires DosS or DosT-mediated phosphorylation for activity.[9,15–17] DosS (one of the two DosR histidine kinases) was induced at the two growth phases tested (Table 1), thus likely activating DosR. We have no formal evidence that increasing DosS transcription would automatically mean that phophorylated DosS and phosphorylation activity of DosR is enhanced, other than the idea that by having more DosS, stochastically DosR-P would augment too. On the other hand, in *Escherichia coli*, a histidine kinase-independent mechanism to phosphorylate (via acetyl phosphate) the transcriptional regulator OmpR has been documented.[18] BCG conserves genes (*ackA* and *pta*)[19] involved in acetyl phosphate production, and although this remains to be formally proven, it is tempting to speculate that acetyl phosphate could be an additional source for activating DosR under aerobic conditions. In *Streptomyces coelicolor*, morphological differentiation events could be activated independently from the normal developmental cascade by overexpression of the transcriptional regulator RamR,[20] thus suggesting that an increase of the number of activator molecules somehow results in their ability to regulate their cognate genes.

In order to assess whether induction of DosR-regulon genes at the transcriptional level resulted in augmented protein synthesis, we used the DosR-regulated, 16 KDa alpha crystallin protein (Acr, HspX)[1] as a marker, monitoring its expression by Coomasie-stained SDS-PAGE, and western blot performed with monoclonal antibody CS-49. A protein band migrating close to 15 KDa was present in BCG::DosR whole cell extracts from from log and stationary phase cultures, and absent from both the parental empty WT and BCG:: pMV361

(Figure 2A). This was confirmed to be HspX by western blot (Figure 2B). The lack of correlation between transcription and translation of HspX could be explained, on the one hand, on the basis of the low protein synthesis rate estimated in mycobacteria even during logarithmic phase, 4.5 fg/bacteria/h.[21] It might well be that we simply do not have enough protein to be detected at OD 0.2. On the other hand, as the cultures were allowed to progress for removing aliquots at OD0.4 and 0.8, it is possible that some of the HspX already translated remains stable and is detected in more dense cultures.

In summary, we have shown that DosR can be produced and that it is active under aerobic conditions in *M. bovis* BCG, with such induction promoting increased transcription of DosR-regulon genes, including the latency antigens *Rv1733c*, *Rv2029*, *Rv2627*, and *Rv2628*, as well as augmented protein synthesis of alpha crystallin, thus suggesting that a recombinant BCG strain expressing DosR under the control of a strong promoter could be a valuable candidate for a latency-specific vaccine.

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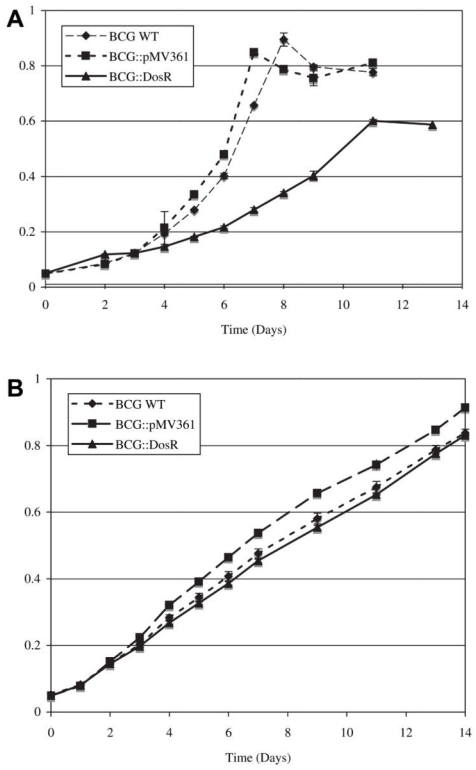

Figure 1.

Aerobic expression of DosR delays the growth of *Mycobacterium bovis* BCG. Growth was monitored by reading optical density at 600 nm of bacteria cultivated at 37 °C in shaken (A) or static (B) 7H9 liquid media supplemented with ADC and 0.05% Tween 80.

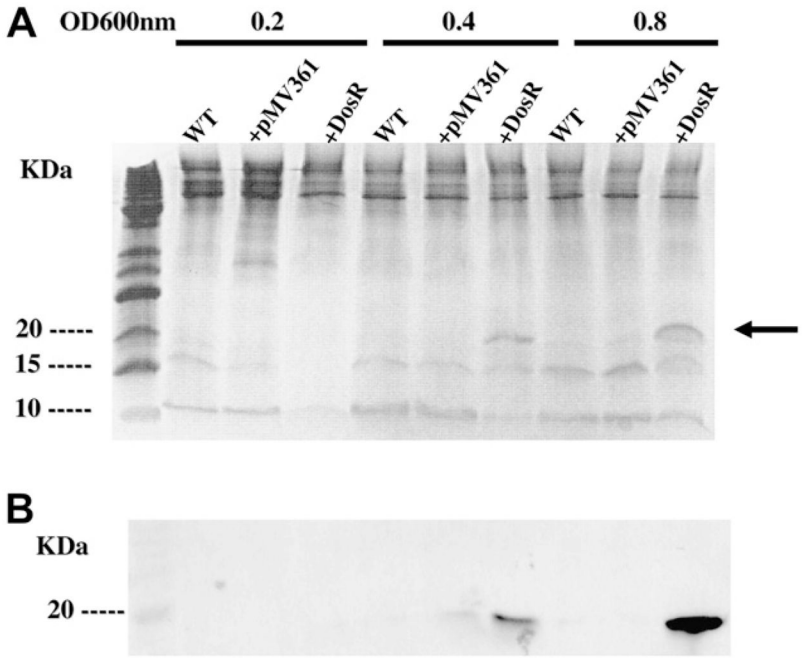

Figure 2.

Aerobic expression of DosR in BCG induces alpha crystallin (Acr, HspX) production. BCG strains containing no plasmid (WT), the vector (+pMV361), or expressing DosR under *hsp60* (+DosR) were grown as described in Figure 1, and at the indicated $OD_{600\ nm}$ samples were stored for lysis. 10 μg of whole-cell extract protein per lane were run in 15% SDS-PAGE and (A) stained with Coomasie blue or (B) transferred to nitrocellulose membrane for blotting with anti-HspX monoclonal antibody CS-49. The arrow indicates a protein band migrating between the 15 and 20 KDa molecular weight markers. This was present exclusively in samples where DosR was expressed.

Table 1

Expression values of DosR-regulon genes in *M. bovis* BCG strains compared to the parental, wild type, non-transformed BCG.

Gene	$OD_{600\ nm}$ 0.2		$OD_{600\ nm}$ 0.4	
	BCG::pMV361	BCG::DosR	BCG::pMV361	BCG::DosR
Rv0079	0.9	11.9	1.2	11.3
Rv0080	1.0	4.3	0.9	3.5
Rv0081	0.9	2.2	1.1	1.9
Rv0569	0.9	27.1	1.0	18.1
Rv0570 (*nrdZ*)	1.0	5.4	1.0	4.7
Rv0571c	1.0	1.1		
Rv0572c	1.1	5.5	1.0	5.4
Rv0573c	1.0	1.7	1.0	2.0
Rv0574c	1.1	1.6	1.0	2.1
Rv1733c	1.0	30.8	1.0	22.8
Rv1734c	1.0	1.5	0.8	1.6
Rv1735c	0.9	1.7	1.0	1.1
Rv1736c (*narX*)	1.0	1.0	0.8	0.8
Rv1737c (*narK2*)	1.0	1.0	0.8	0.9
Rv1738	1.0	22.7	1.0	16.1
Rv1739c	1.0	0.9	1.0	1.0
Rv1812c	1.0	7.2	1.0	8.4
Rv1813c	0.9	43.5	1.0	27.9
Rv1996	1.0	20.3	0.9	21.4
Rv1997 (*ctpF*)	1.0	23.6	0.9	15.4
Rv2003c	0.9	7.0	0.9	6.7
Rv2004c	1.0	9.8	0.9	7.6
Rv2005c	0.9	14.2	1.0	12.1
Rv2006 (*otsB*)	1.0	5.0	1.0	3.8
Rv2007c (*fdxA*)	0.9	26.4	1.1	19.6
Rv2028c	1.0	9.8	1.0	13.6
Rv2029c (*pfkB*)	1.1	26.3	1.1	19.7
Rv2030c	1.1	68.2	0.7	
Rv2031c (*acr, hspX*)	0.8	57.0	1.2	35.6
Rv2032	1.0	48.0		
Rv2033c	1.1	1.7	1.1	1.3
Rv2623	0.9	3.6	1.0	3.1
Rv2624c	1.0	10.1	1.3	6.5
Rv2625c	0.9	9.7	0.8	
Rv2626c	1.0	51.4	1.2	29.4
Rv2627c	0.9	43.8	1.0	32.3
Rv2628	1.0	36.6	1.1	29.5

Gene	$OD_{600\ nm}$ 0.2		$OD_{600\ nm}$ 0.4	
	BCG::pMV361	BCG::DosR	BCG::pMV361	BCG::DosR
Rv2629	0.9	6.9	1.0	6.4
Rv2630	0.9	8.9	1.0	8.5
Rv2631	0.9	3.0	1.0	2.8
Rv3126c	1.0	6.0	3.0	
Rv3127	0.9	35.1	24.5	
Rv3128c	0.9	1.7	0.9	1.3
Rv3129	1.0	1.3	0.9	1.3
Rv3130c	1.0	18.7	1.0	14.9
Rv3131	1.0	61.2	1.0	43.8
Rv3132c (*dosS*)	1.1	11.3	1.2	14.2
Rv3133c (*dosR*)	1.1	16.4	1.1	12.6
Rv3134c	1.0	38.3	1.0	33.2

Empty spaces indicate data values not available.

Table 2

Expression values of DosR-regulon genes in *M. bovis* BCG compared to *M. tuberculosis* H37Rv Δ*Rv3134c-3132c* (*Rv3134c-dosRS*) knockout.

Gene	Fold-change
Rv0079	11.8
Rv0080	3.2
Rv0081	3.7
Rv0569	14.3
Rv0570	4.9
Rv0571c	2.2
Rv0572c	6.3
Rv0573c	2.0
Rv0574c	2.7
Rv1733c	18.0
Rv1734c	1.2
Rv1735c	1.5
Rv1736c	0.9
Rv1737c	1.2
Rv1738	24.1
Rv1739c	1.6
Rv1812c	12.3
Rv1813c	15.5
Rv1996	28.4
Rv1997	14.3
Rv2003c	6.9
Rv2004c	16.1
Rv2005c	13.1
Rv2006	3.5
Rv2007c	15.7
Rv2028c	28.4
Rv2029c	25.3
Rv2030c	50.1
Rv2031c	30.9
Rv2032	22.2
Rv2033c	2.1
Rv2623	3.9
Rv2624c	6.4
Rv2625c	7.6
Rv2626c	35.1
Rv2627c	27.3
Rv2628	40.7
Rv2629	13.2

Gene	Fold-change
Rv2630	15.7
Rv2631	2.9
Rv3126c	3.4
Rv3127	24.1
Rv3128c	1.8
Rv3129	2.3
Rv3130c	46.9
Rv3131	38.1
Rv3132c	29.1
Rv3133c	19.4
Rv3134c	56.6